The feasibility of a commercial-scale recirculating aquaculture system integrating sea urchins (*Tripneustes gratilla*) and seaweed (*Ulva*).

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Declaration

This thesis has not been submitted in this or any form to another University. Experimental work discussed in this thesis was carried out under the supervision of Prof. J. J. Bolton of the Department of Biological Sciences, University of Cape Town Dr Mark D. Cyrus of the Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Australia and Dr Brett M. Macey, from the Department of Fisheries, Forestry and Environment, South Africa. I declare that this thesis is my own, unaided work besides that specified. This includes the histology of urchin gonads conducted by the Boland State Veterinary laboratory, Elsenberg, Stellenbosch. The nutritional analysis of *Ulva lacinulata* was conducted by Nvirotek, Wellington. Lastly the water quality analyses were conducted by the Marine Biogeochemistry Lab in the Department of Oceanography at the University of Cape Town. Specifically where Raymond Roman measured the nitrate, nitrite, silicate, and phosphate concentrations, Hazel Leighton-Little measured the ammonium concentrations and Sarah Fawcett quality controlled the data. The use of the equipment was allowed for by the University of Cape Town Equipment Committee and supported by The South African Department of Science and Innovation's Biogeochemistry Research Infrastructure Platform (BIOGRIP).

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Abstract

It has been proposed that the highly valued sea urchin, *Tripneustes gratilla*, and the green macroalga, *Ulva*, could be suitable co-candidates for a land-based recirculating Integrated Multi-Trophic Aquaculture (IMTA) system. This IMTA system is suggested to be sustainable and efficient, primarily because the *Ulva* would simultaneously provide bioremediation of effluent and a substantial feed source for *T. gratilla*. While evidence for these proposed benefits does exist, it is dispersed and incomplete. The primary aim of this thesis is to create a farm-scale model to provide clear evidence of the biotechnical feasibility of a *T. gratilla-Ulva* IMTA system. To develop this model, literature gaps needed to be filled. These gaps became the auxiliary initial objectives, specifically; to determine optimal *T. gratilla* basket depth, stocking density and quantification methodology to allow for the accurate prediction of *T. gratilla* nitrogen emissions.

The literature indicates that production of various urchin species is reduced when cultivated in deeper baskets, in contrast to shallower baskets. Thus, if and why basket depth has this effect was investigated and an ideal basket depth for *T. gratilla* culture was determined. Deeper baskets (30 cm deep) resulted in significantly lower consumption of various feed types (formulated feed, *Ulva lacinulata* and *Ecklonia maxima*; $W > 38$, $p < 0.026$). This is likely the consequence of lower feed accessibility, which in turn causes the observed reduced yield. Therefore, shallow baskets (±15 cm deep) enhance production of *T. gratilla* and were applied in subsequent stocking density trials.

The stocking density of *T. gratilla* expected in commercial systems has not been clarified or optimised. Two trials were conducted where production parameters were compared between urchins stocked at various densities. The first trial was a three-month grow-out trial where only fresh *Ulva lacinulata* was supplied and the objective was to maximise urchin size. The subsequent trial focused on maximising gonad production where predominantly a formulated feed with a 20% *Ulva* inclusion was provided over two-months. While higher stocking densities did significantly reduce the specific growth rates of average individual urchin mass in both trials ($p < 0.044$), there was no indication that mortality, cannibalism or gonad size and quality was influenced by the densities tested in this study. Thus, it was concluded that the optimal stocking density for both the grow-out and gonad enhancement phases of *T. gratilla* is approximately 20% coverage (surface area of urchins' tests by surface area of basket), regardless of urchin size.

During the above trials it became apparent that the implementation of a commercial *T. gratilla* culture would not be feasible in the absence of a reliable and precise method to measure large

quantities of live urchins, which is necessary for the successful development and management of such an industry. Therefore, to avoid this future bottleneck, this study also developed an accessible, accurate and efficient protocol for the reliable and precise measurement of large quantities of sea urchins using computer vision. For a larger-scale context, this open-source software could easily be incorporated into various tools, such as a grading machine, to completely automate farm processes. Additionally, this protocol can be used in a research context to greatly enhance the accuracy and standardisation of live urchin measurements.

The foundation of the *T. gratilla-Ulva* IMTA model is a total ammonia nitrogen (TAN) sub-model, as TAN is the most limiting exchanged resource. Due to the lack of literature on *T. gratilla* nitrogen emissions and its complexity, an empirical 'black box' approach using regression models was used for predicting TAN concentrations in *T. gratilla* effluent. Training and validation data were acquired from an extensive trial where water samples were collected from urchin systems with different treatments hypothesized to influence water quality, such as flow rates, feed type and quantity of urchins. Additionally, additional water quality parameters were observed, thereby providing an in-depth understanding of how *T. gratilla* aquaculture systems will influence water quality. The nitrogen concentration in *T. gratilla* effluent was low by aquaculture standards (average 0.001 mg/l TAN) while the high dissolved CO_2 concentrations were of concern (maximum of 525 µATM). This provides novel insights, from which valuable management recommendations are made.

As the literature gaps were filled as described above, it became possible to create the *T. gratilla-Ulva* farm-scale IMTA model, which would not only assist justifying the integration of these species but also estimate the production capabilities of this system. To ensure its applicability and practicality, the model was developed as a digital twin of the established and extensively validated commercial abalone-*Ulva* IMTA farm systems in South Africa. The model suggests that, based on the proposed monthly culture cycle, the *T. gratilla* capacity of the 42 tanks (8.5 m^3 each) is approximately 360 000 individuals of seven successive cohorts, which could result in a monthly gonad harvest between 0.31 and 0.88 t per month, depending on finishing feed. The model revealed that while the simulated 300 m^2 *Ulva* raceway could remove all the TAN emitted by *T. gratilla* (average of 0.009 mg/l), it cannot be considered efficient as a biofilter as it requires considerably more farm surface area (land footprint) than necessary. The projected TAN emissions from the *T. gratilla* production system are insufficient to sustain the *Ulva* population, let alone enable substantial *Ulva* production for *T. gratilla* feeding. Hence, the farm-scale model indicates there is inadequate nitrogen emissions from the *T. gratilla* production unit to justify the integration of *Ulva* with *T. gratilla* production, based on the existing abalone-*Ulva* IMTA systems. Yet a nitrogen retention model provides evidence that the nitrogen supplied in the urchin feed is sufficient for substantial *Ulva* production. Most of this nitrogen is likely deposited as particulates and thus cannot be utilized by *Ulva*. However, this nitrogen could be converted into a dissolved form via mineralization. While further investigation is required, this indicates the potential to create a circular and efficient IMTA system if designed and managed correctly. Importantly, the model indicates that *T. gratilla* farming using this land-based system would not be limited by ammonia toxicity and would likely be highly productive when compared to aquaculture of other high value species.

Therefore, this study indicates that this recirculating *T. gratilla-Ulva* IMTA system may be feasible if the correct design or management adjustments are made. The recommended basket design, stocking density, quantification method, water quality insights and the farm-scale model could greatly assist establishing this potential industry. Additionally, with further development, the model could be used as a tool for environmental impact assessors, farmers, entrepreneurs and investors to design farms or conduct economic, environmental and social analyses across various scales.

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Chapter 1: General Introduction

1.1 Integrated multi-trophic aquaculture (IMTA)

Aquaculture is the controlled cultivation or farming of aquatic organisms, which plays a vital role in helping to meet the world's ever-growing need for food. When not managed carefully, however, aquacultural practices can have adverse environmental effects. High densities of organisms may result in large outputs of high nutrient effluent, which can pollute surrounding waters (Martinez-Porchas and Martinez-Cordova, 2012). The production of one kilogram of edible fish biomass from aquaculture can require up to five kilograms of wild fish for use as feed (Naylor *et al.*, 2009), thus contributing substantially to overfishing. Aquaculture has been among the primary causes of the introduction of invasive species (Garibaldi and Bartley, 1998; Naylor *et al.*, 2001). These exotic species can become invasive, disrupt ecosystems and introduce new diseases (Savini *et al.*, 2010). Aquaculture has also resulted in habitat destruction. This includes the loss of 544 000 ha of mangroves globally (accounting for 28% of the total loss; Hamilton, 2013). The aquaculture also contributes to greenhouse-gas (GHG) emissions through various pathways (MacLeod *et al.*, 2020). Among these are feed production and blue carbon storage loss, where shrimp, for example, can have a land-use carbon footprint even greater than beef (Kauffman *et al.*, 2017). These are just a few examples of the adverse impact aquaculture can have on the environment. Nevertheless, the practice is a vital method of food production, and all forms of agriculture can have negative environmental impact if managed incorrectly.

With the human population recently reaching eight billion, the efficient and sustainable production of food is a priority. Seafood is essential for human nutrition, accounting for 17% of the global animal-derived protein consumed in 2018 (FAO, 2020). Furthermore, seafood has been consumed since the origins of man (Klein, 2001) and thus has a strong cultural significance across the globe (Fabinyi, 2012; MacDonald *et al.*, 2015; Shamsi *et al.*, 2020). The ever-growing human population, along with the nutritional and cultural importance of seafood, means there will be a continuous, burgeoning demand for seafood. This demand has not and will not be sustained by traditional capture fisheries (Fig. 1.1). Aquaculture must continue filling the gap between capture fisheries production and global demand for seafood. At present, the industry is at a transitional phase, during which time aquaculture recently surpassed capture fisheries as the primary source of seafood and is recognised as the fastest growing agriculture sector globally (FAO, 2021). This has been compared to the agricultural revolution that occurred at least 10 000 years ago (Solheim, 1972), when humans transitioned from hunting and gathering to (terrestrial) farming.

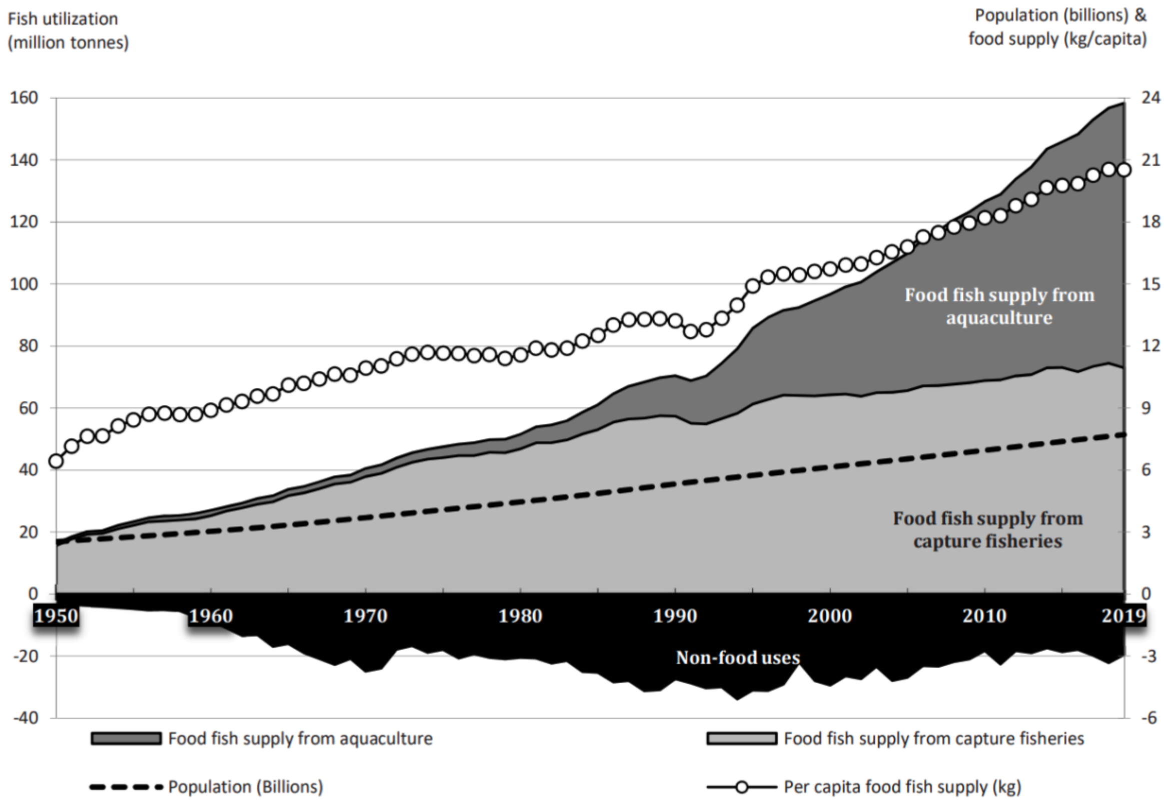


Fig. 1.1. A comparison of sources of global supply of seafood (labelled as fish) relative to population growth and consumption. Graph was sourced from the FAO Fishery and Aquaculture Statistics 2021 Yearbook. (https://www.fao.org/fishery/static/Yearbook/YB2019_USBcard/booklet/web_cb7874t.pdf)

The extraordinary growth of aquaculture to fulfil demands that cannot be met by capture fisheries production is concerning some people because of its potential impacts on the environment (Martinez-Porchas and Martinez-Cordova, 2012). Crucially though, aquaculture is not inherently unsustainable and, where managed correctly, can even benefit the environment (Alleway *et al.*, 2019; Chopin *et al.*, 2012; Klinger and Naylor, 2012). Many hypothesize that production from aquaculture will reduce the pressure on wild stocks and their ecosystems (Anderson, 1985; Frankic and Hershner, 2003; Valderrama and Anderson, 2010). Others argue the opposite, saying that aquaculture might supplement wild capture fisheries due to reasons such as aquaculture's demand for wild fish as feed or feed ingredients (Longo *et al.*, 2019; Naylor *et al.*, 2000). The debate will probably endure but, regardless of whether aquaculture offsets capture fisheries production, the demand for seafood will remain. What is important is that aquaculture meets this demand without the severely damaging impact it has had on the environment in the past. Consequently, the development of sustainable aquaculture techniques has been a focus of academia, industry, and governments globally for decades with considerable progress being made. This has seen the introduction of many solutions to advance the sustainability of aquaculture. While no single solution will achieve this, there is one specific concept gaining great momentum globally as it addresses and mitigates many of the sustainability and production issues faced by aquaculture. This concept is known as integrated multi-trophic aquaculture (IMTA).

IMTA was recently defined as “a concept where different species are grown together in such a way that the invertebrates and/or plants can recycle the nutrients that are lost from the culture of the other species” (European Union, 2020). While the name IMTA was only coined in 2004 (Chopin and Robinson, 2004), the method has been used successfully in Asia for centuries (Li, 1987; Liao, 1992; Qian *et al.*, 1996). Integrated multi-trophic aquaculture can be applied to most forms of aquaculture, from freshwater ponds to offshore oceanic systems. As such, the ways it can reduce impact and enhance efficiency are highly diverse and depend on the specific form it takes. The primary advantage of IMTA is, however, consistent across all forms, namely the utilisation of by-products or waste from the fed organism to sustain one or more co-produced organisms from lower trophic levels.

An example that demonstrates the rationale behind IMTA and the motive for this thesis is the commercial-scale, land-based abalone-*Ulva* IMTA systems established in South Africa (Fig. 1.2). *Haliotis midae* is an abalone species endemic to South Africa and highly valued in Asian markets. Overexploitation of wild stocks and their high value led to the establishment of the South African abalone aquaculture industry in 1981 (Sales and Britz, 2001). Most farms had access to kelp beds, *Ecklonia maxima*, and therefore it was used as a primary feed source for the abalone. Although at that time, two farms did not have access to kelp and therefore began experimenting with alternative macroalga feeds by culturing a red seaweed *Gracilaria* spp. in the effluent of the abalone tanks during the early 1990s and then, in the early 2000s, with the green seaweed *Ulva* spp. (Bolton *et al.*, 2009; Steyn, 2000). The *Ulva* was found to be highly suited to this role. At present, multiple farms, including many with access to kelp, have integrated the cultivation of *Ulva* with abalone.

Fig. 1.2. A commercial recirculating land-based abalone-*Ulva* integrated multi-trophic aquaculture system in Buffeljags, South Africa. Here, *Ulva lacinulata* is cultivated in 28 paddle raceways (approximate surface area of 300m^2 each) using effluent of *Haliotis midae*, which are grown in more than 1 100 tanks (8.5m^3 volume each) resembling cells adjacent to the *Ulva* ponds. The *Ulva* provides both feed and bioremediation for the abalone. Source: www.vikingaquaculture.co.za

Ulva grows at remarkably high rates in paddle-raceways (±22.9 g m^{-2} day^{-1} dry weight (DW); Bolton *et al.*, 2009). The annual production in South Africa is at least 2 000 t wet weight (WW) (Bolton *et al.*, 2016; FAO, 2022; Neveux *et al.*, 2018). This *Ulva* is used as a supplementary feed for *H. midae* (Naidoo *et al.*, 2006) and has been shown to increase consumption and improve abalone condition (Dlaza *et al.*, 2008). As such, the original objective to provide an alternative feed source for abalone was successfully achieved through IMTA. This, however, was found to be just one of many benefits of including *Ulva* in these systems. Several of the observed benefits are the result of the biofiltration capability of *Ulva*, which removes nutrients (or abalone waste products), thus allowing for partial recirculation of water instead of the traditional pump-ashore, flow through, system (Bolton *et al.*, 2016). It has been estimated that the integration of *Ulva* on a single farm will reduce the annual quantity of nitrogen emitted into the ocean by up to five tonnes, reduce annual kelp harvest by up to 6.6 ha and GHG emissions by up to 350 t CO_2 (Nobre *et al.*, 2010). The benefits are not only environmental. Onsite feed production and reduced pumping requirements (therefore, less energy consumption) mean the profitability of the farm increases by up to 5% (Nobre *et al.*, 2010). Additionally, the bioremediation offered by the *Ulva* extends beyond nutrient removal as it also modulates the microbiome, which enhances abalone production (de Jager, 2021; Macey *et al.*, 2022). Furthermore, the risk of losing abalone stock is also significantly reduced when harmful algal blooms (HABs) occur in the vicinity of the farm. HABs are a major threat to the South African abalone aquaculture industry. In January 2017, a minimum of 250 t of abalone were lost due to a dinoflagellate bloom in Walker Bay, South Africa, where a large majority of commercial abalone farms are located (Pitcher *et al.*, 2019). In such cases, the biofiltration ability of *Ulva* allows farms to completely recirculate their water for a few days, thereby isolating themselves from the surrounding environment and potentially avoiding uptake of the seawater containing the HAB.

At present, South Africa produces the most abalone outside Asia (Cook, 2019). Approximately 96% of South Africa's aquaculture revenue is derived from the land-based cultivation of abalone (*Haliotis midae*) (DAFF, 2016). While the success of the industry cannot be contributed entirely to IMTA, the abalone-*Ulva* IMTA farms are important in a global context. They are among the few examples of successful commercial-scale IMTA outside East Asia, despite other regions having committed considerable research and funding to the endeavour. The success of the abalone-*Ulva* IMTA systems in South Africa triggered interest in creating similar systems for other species, including, as is the focus of this thesis, the use of *Ulva* with other primary or fed organisms such as sea urchins.

1.2 *Ulva*

To explicate the ever-increasing interest *Ulva* attracts across disciplines globally, the year 2022 saw an entire conference, which is a part of a four-year European Union Cooperation in Science and Technology (EU COST) project, dedicated to this single genus. The SeaWheat Conference took place in Spain in September 2022 and was so named as a branding attempt to associate this green macroalgae with wheat (*Triticum* sp.). This is to indicate the prospective shared characteristics of *Ulva* and wheat, where both are highly productive and versatile crops suited to mass production. Despite some allegations of hype in the algaculture industry (Ganesan *et al.*, 2020), there is considerable evidence that *Ulva* could be a significant source of sustainable and useful biomass, with additional beneficial characteristics, such as biofiltration.

The biology of *Ulva* reveals its versatility, plasticity and suitability to aquaculture. This green macroalgae genus is from the *Chlorophyta* phylum. It is found in fresh, brackish and marine waters and occurs in polar to tropical environments (Tran *et al.*, 2022). It is commonly known as sea lettuce due its typically flat (only two cells thick) and leafy appearance (Fig. 1.3). However, it is polymorphic, taking many forms, including the tubular and filamentous (Mantri *et al.*, 2020). The morphologies are regulated by species, salinity (Rybak, 2018) and symbioses with bacteria (Wichard *et al.*, 2015). Various species of this genus grow in a variety of manners such as attached, sessile or free floating (Mantri *et al.*, 2020). This means it can be grown successfully using multiple culture methods, including land-based ponds, paddle raceways or attached to ropes in the sea (Bolton *et al.*, 2009; Carl *et al.*, 2014). The phenotypic plasticity of *Ulva* can be useful for its production but causes problems for its taxonomy (Bolton, 2019). Currently, there are 102 *Ulva* species accepted taxonomically (Guiry and Guiry, 2022). However, the name *Ulva lactuca* is falsely applied to a majority of *Ulva* used in aquaculture (Bolton, 2019). There is strong evidence that all IMTA farms in South Africa have the same single species; *Ulva lacinulata* (Bachoo, 2021; Fig. 1.3). While the plasticity of *Ulva* is problematic in terms of taxonomy and nomenclature, numerous advantages arise from the biology of *Ulva*, specifically for aquaculture.

Fig. 1.3. A free-floating thallus of *Ulva lacinulata*, which is farmed commercially in South Africa using raceways fed effluent from *Haliotis midae* (abalone) tanks.

The nutritional content of *Ulva* also varies widely depending on conditional factors, such as water chemistry and light (Dudley *et al.*, 2010; Olsson *et al.*, 2020). Protein content has been observed to be as low as 10% and as high as 47% (Fleurence, 1999; Kraan, 2013). Protein sources in aquaculture feed are understood to be the costliest feed components, both financially and environmentally (Jia *et al.*, 2022). As such, *Ulva* with high protein can be highly valuable as a feed or feed ingredient/protein source.

The reproduction processes of *Ulva* can be either fragmentation of thallus or by microscopic propagules (spores or gametes). The fragmentation method has been most effectively utilized in land-based aquaculture because of its simplicity, where, for example, it does not require specific cues to induce sexual reproduction as it propagates primarily through vegetative reproduction (personal observation). Sea-based *Ulva* aquaculture, on the other hand, relies on the production of spores or fertilised gametes that settle onto ropes suspended in the ocean (Steinhagen *et al.*, 2021).

The productivity and growth rate of *Ulva* is so high that, if left uncontrolled in certain nutrient-rich habitats, it can cause environmental issues. In the ocean, *Ulva* has been known to attain enormous biomass sporadically and rapidly, sometimes to the point of dominating marine ecosystems. When this occurs, the phenomenon is referred to as a green tide (Fig. 1.4). It can lead to biodiversity loss through habitat loss, hypoxia, sulphur emissions and acidification (Deegan *et al.*, 2012; Han *et al.*, 2021; Hu *et al.*, 2015; Lyons *et al.*, 2014; Zhang *et al.*, 2019). Green tides occur due to anthropogenic eutrophication and proliferation of fast-growing strains in affected areas (Fort *et al.*, 2020). *Ulva*'s ability to grow at high rates can, however, be highly advantageous where controlled, such as in aquaculture.

Fig. 1.4. A green tide of *Ulva* in Qingdao, China 2013, indicating *Ulva*'s remarkably high growth rate in ideal conditions. Source: El País 14/7/2013

Ulva grown in commercial raceways with abalone effluent has yielded approximately 83.59 t DW.ha^{-1}.y^{-1} (Bolton *et al.*, 2009). Another study suggests yields of 45 t DW.ha^{-1}.y^{-1} are viable (Bruhn *et al.*, 2011). For context, maize (*Zea mays*) is the grain crop with the highest yield per hectare and from 2017 to 2019 had an average global yield of 5.8 t DW.ha^{-1}.y^{-1} (Erenstein *et al.*, 2022). *Ulva*'s high growth rate also promotes a low susceptibility to epiphytism (Bulboa *et al.*, 2007; Jiménez del Río *et al.*, 1996; Mata and Santos, 2003; Msuya and Neori, 2002), which can reduce quality and yield in algaculture (Ask and Azanza, 2002; Fletcher, 1995; Oliveira *et al.*, 2000). To support the high growth rates of *Ulva*, large quantities of nutrients, such as nitrogen and phosphate, are required (Solidoro *et al.*, 1997). These nutrients are frequently the waste products of various processes, including aquaculture. This makes *Ulva* a highly effective biofilter (Bolton *et al.*, 2016; Jiménez del Río *et al.*, 1996; Mata *et al.*, 2010; Neori *et al.*, 1996; Yokoyama and Ishihi, 2010), specifically for ammonia removal (Ale *et al.*, 2011). This is beneficial because ammonia is generally considered the first nutrient to accumulate in aquaculture systems and become toxic, thereby reducing production (Hargreaves, 1998).

While the potential of *Ulva* extends well beyond its suitability as feed for aquaculture livestock and bioremediation of aquaculture effluent, the success of its integration with *H. midae* has inspired ideas for combining it with other fed aquaculture species. One potential candidate that might be suited for co-cultivation with *Ulva* is the sea urchin *Tripneustes gratilla*, as per the focus of this study.

1.3 *Tripneustes gratilla*

Tripneustes gratilla (Fig. 1.5) is a tropical/sub-tropical sea urchin species. Found throughout the tropical Indo-West Pacific, from South Africa to Hawaii, including the Red Sea, extending into subtropical regions in e.g. South Africa and Australia (Cyrus, 2013; Mortensen *et al.*, 1943), *T. gratilla* is well suited to aquaculture. Unlike most of the other higher-value urchin species, which are generally found in temperate waters, it has a fast growth rate and has been reported to attain harvest size and sexual maturity within five to six months (Shpigel *et al.*, 2018). Furthermore, both sexes are harvestable and can have large marketable gonads, often 20-25% of the total body mass (Cyrus, 2013).

Fig. 1.5. A wild adult *Tripneustes gratilla* sea urchin collected in Mgazana, Eastern Cape, South Africa.

The value of *T. gratilla* lies in these gonads. Sea urchin gonads, also known as 'uni', are a demanded and valued culinary delicacy. Japan has the largest sea urchin market, annually consuming approximately 50 000 t (Stefansson *et al.*, 2017). Global overexploitation of wild sea urchins has, however, resulted in a dramatic decline of natural populations, with many stocks having completely collapsed (Andrew *et al.*, 2003). Sea urchin farming or echinoculture is regarded as a more sustainable solution and the only way to meet future global demand for urchin-related products.

Tripneustes gratilla was first documented as a highly suitable candidate for aquaculture more than four decades ago (O'Connor *et al.*, 1976). Since then, some 1 200 papers have been published about their culture (Google Scholar, 2022). After all this time and research, there are currently no clear reports of active commercial, closed life-cycle aquaculture systems for this echinoderm. There are few cases of practical culture operations using this species. These include some small reseeding projects in Japan (Unuma *et al.*, 2015), community-based grow-out and restocking in the Philippines (Bell and Garces, 2004; Juinio-Meñez *et al.*, 2008), and biological control for invasive seaweeds in Hawaii (Conklin and Smith, 2005). In Australia, *T. gratilla* has been farmed from embryo to adult on a pilot scale by AusUni Pty Ltd. The company began building a commercial-scale production facility, however, this was aborted due to the global recession in 2010 (Brown and Eddy, 2015).

The lack of commercial production of this urchin species might seem surprising. *T. gratilla* appears to hold a higher value and superior productivity than *H. midae*, which is the mainstay of the South African aquaculture industry. *T. gratilla* can achieve market size (*ca.* 100 g) in five to eight months (Cyrus *et al.*, 2015a; Shpigel *et al.*, 2018), while *H. midae* reaches market size in about five years. Abalone has a farm gate price of between R500 to R840 per kg (Antoni, 2018). The farm gate price of *T. gratilla* is not known but the value for top quality urchin gonads in Japan can be R4 000-R9 000 per kg (Sonu, 2003; "Tsukiji-Market," 2016). There does not appear to be a lack of knowledge about the culture methods of *T. gratilla*, and full lifecycle grow-out trials (from fertilization to marketable adult urchins) have occurred in multiple facilities globally. The lack of commercial production of this urchin species therefore suggests a discrepancy between academia and industry, which is preventing the effort and resources put into the research from being applied commercially.

There is anecdotal evidence that an urchin-*Ulva* IMTA system will be functionally, environmentally and financially viable, as implied through large amounts of literature as mentioned in the preceding text. Particularly relevant here is that some of the highest recorded growth rates and gonadosomatic indexes (GSI) are the result of feeding a diet of either fresh *Ulva* or pellets with *Ulva* as a primary component (Cyrus *et al.*, 2015a, 2015b; Shpigel *et al.*, 2018). There is, however, a lack of consolidated and quantitative evidence to validate this novel farming system and whether this species combination will be feasible. This is likely a primary reason for the lack of adoption by industry. It is clear *Ulva* will provide feed and bioremediation for *T. gratilla*. However, it is not clear if the extent of these services will warrant the resources required to co-culture *Ulva*. Furthermore, for this recirculating IMTA system to realise its intended benefits, a fine balance of resources must be achieved between the fed (urchins) and extractive (*Ulva*) species. For example, the waste/nutrients produced by the urchins must be assimilated by the *Ulva*. If there is insufficient assimilation of waste, it will reach toxic levels and reduce urchin production. Although, if there are not enough nutrients emitted by the urchins, it will not support growth or survival of the *Ulva*. This balanced is achievable through establishing appropriate conditions via system design and management, however, these appropriate conditions have not been defined for a *T. gratilla*-*Ulva* IMTA system. Therefore, to justify the inclusion of *Ulva*, achieve the balance between organism and facilitate the adoption of commercial urchin-*Ulva* IMTA, potential future stakeholders need to be able to estimate the inputs (such as feed, number of tanks, required flow rates) and outputs (such as nitrogen content in effluent and yield of urchin gonads) of the urchin-*Ulva* IMTA system. Without determining these inputs and outputs, the functional, environmental and/or economic feasibility of this IMTA system cannot be determined. This is preventing the initiation of this new aquaculture sector. What is the solution to this?

1.4 Farm-scale modelling

It is not realistic to test the new species combination in a full-scale physical system. The existing abalone-*Ulva* farms in South Africa could not be used to test the urchin-*Ulva* combination for numerous reasons. Firstly, it is not yet known if it is practically possible to farm urchins in systems used to farm abalone. The water chemistry balance between the *Ulva* and urchins is unknown. What is understood is that it is unlikely to be that same as that seen in the abalone-*Ulva* systems. Different species have very different influences on water chemistry (Dy and Yap, 2000). *Haliotis midae* and *T. gratilla* production require different water temperatures and this will impact *Ulva*'s effect on water chemistry (Solidoro *et al.*, 1997). Furthermore, existing abalone farms are largely located in areas where water temperature is too low for *T. gratilla* production. Heating could be an economic limitation. Additionally, even if an abalone farm could be used to test urchin production, the market channels have not been established. Full-scale testing of *T. gratilla* production at existing abalone facilities would put them at risk of suffering severe financial losses, particularly regarding opportunity loss of abalone production. To build a new farm in a temperature appropriate site would require significant capital investment. Aquaculture, especially where a new species is involved, is known to be a high-risk investment (Kumar *et al.*, 2018; Pomeroy and Getchis, 2003), which means that without clarity on the aforementioned issues, it is unlikely that capital for such a project would be forthcoming.

If it is not possible to test urchin-*Ulva* IMTA systems at existing facilities, how could feasibility be trialled without building a new system? The establishment of a pilot system is a good option. In fact, a *T. gratilla*-*Ulva* IMTA pilot system is currently operating on site at a commercial scale abalone-*Ulva* IMTA farm at Buffeljags in the Western Cape, South Africa (Fig. 1.2). Once this system is fully stocked with *T. gratilla*, it will provide strong insight into the feasibility of the system. There are, however, some limitations. Aquaculture is known to present a scaling issue (Badiola *et al.*, 2012; Castex *et al.*, 2014; Macaulay *et al.*, 2021; Ngo *et al.*, 2017; Troell *et al.*, 2003; Van Den Hende *et al.*, 2014), meaning the performance or responses of a specific aquaculture system may not be constant or linear. For example, if a hypothetical pilot system of five urchin tanks and a small *Ulva* raceway shows good performance, it does not necessarily mean 40 urchin tanks with a large *Ulva* raceway will work. This inconsistency in scale means the knowledge gained from the pilot system is not necessarily entirely transferable. Furthermore, a pilot system itself will not allow for easy optimisation of the future systems. This is particularly relevant if only production metrics (such as growth rates) of the pilot system are observed, and the driving forces or limitations of production (such as water chemistry) are not. While pilot systems are undoubtably a necessary step in the development of a new form of aquaculture, the issues of scalability, optimisation and transferability mean more needs to be done to fully assess the functionality of a novel system.

There is a solution. A highly efficient method to determine feasibility of such a system is through farm-scale digital simulation modelling (Cacho, 1997; Chary *et al.*, 2022; Reid *et al.*, 2020). This is subsequently the primary approach used in this study. A simulation model provides extensions of general models, such as those used for hypothesis testing. The simulation model constructed in this thesis will create and provide an analysis of a digital prototype of a full-scale urchin-*Ulva* system to predict its performance. In engineered and controllable ecosystems, such as land-based recirculating aquaculture, simulation models can provide a wide variety of additional uses (Cacho, 1997). They can offer valuable insight into the functional, environmental and economic feasibility and provide opportunities for in-depth optimisation. All of this is possible without building a physical system.

An example of this is a study that used modelling to assess the feasibility of using sea cucumbers to mitigate the effluent from finfish (*Sciaenops ocellatus*) held in cages (Chary *et al.*, 2020, 2019). There is considerable discussion and evidence in the literature indicating the potential of sea cucumbers as highly suitable candidates in IMTA (Zamora *et al.*, 2018). This is partly due to their ability to feed on detritus, thus providing bioremediation of effluent (Zamora *et al.*, 2018). This study though, found that in this particular form of aquaculture system, the sea cucumbers were not a suitable candidate for the objective of bioremediation as they only removed an inconsequential amount (0.73%) of the particulate fish waste (Chary *et al.*, 2020). This was determined via modelling using the data in existing literature without any additional physical experimentation. As such, there were minimal wasted resources and risk to the environment.

This thesis applies a similar approach to determine the feasibility of land-based and recirculating *T. gratilla* and *Ulva* aquaculture. As previously stated, a balance between the *Ulva* and urchins must be attained to achieve the proposed benefits of this recirculating IMTA system. The primary limiting resource for both the *Ulva* and urchins will likely be total ammonia nitrogen (TAN) as implied by the literature (Hargreaves, 1998; Lavery and McComb, 1991; Siikavuopio *et al.*, 2004b; Solidoro *et al.*, 1997), discussed further in Chapter 5. Therefore, if it can be determined that an *Ulva* raceway is capable of assimilating the TAN emitted by an urchin production system, it will indicate if the IMTA system can achieve this balance. Where, if it indicates the balance is not achieved, this would show what conditions (such as flow rates, volume of tanks and biomass ratios) would be required to achieve it. This nitrogen mass balance model will be used to provide the foundations of a production model, thereby allowing the yields of both *Ulva* and *T. gratilla* to be estimated. A simulation model could provide estimates into both the biofiltration and production capacities, thereby assessing the functional feasibility of this conceptual IMTA system.

The urchin-*Ulva* IMTA model requires extensive quantitative information to create the sub-models that would be merged to create the complete model. Fortunately, much of the required data and even certain sub-models are available in the literature, especially for *Ulva* (Ben-Ari *et al.*, 2014; Bolton *et al.*, 2016, 2009; Oca *et al.*, 2019; Shpigel *et al.*, 2019, 2018; Solidoro *et al.*, 1997; Troell *et al.*, 2006). While there is considerable information regarding *T. gratilla* (Cyrus *et al.*, 2015, 2014, 2013; Dafni, 1992; Shpigel *et al.*, 2018; Shpigel and Erez, 2020), it was, however, determined that there were some literature gaps that would inhibit the synthesis of this model. Closing these literature gaps emerged as the additional objectives and chapters of this thesis.

1.5 Aims and objectives

The aim of this thesis is to assess the feasibility of a recirculating, land-based *Tripneustes gratilla* system integrated with *Ulva*. This will be done via a farm-scale model. However, prior to this, it was necessary to fill-in specific literature gaps that prevented the synthesis of this model and/or would help remove bottlenecks in commercial urchin production. Specifically, these additional aims of this thesis were to:

I. Determine the influence of basket depth on the production of *T. gratilla;*
II. Suggest an optimal stocking density for *T. gratilla* production;
III. Develop a protocol to measure live urchins with precision and efficiency on a large scale; and
IV. Determine the influence *T. gratilla* has on water chemistry in an aquacultural context.

The justification of these aims and more details are as follows;

I. Prior to suggesting an optimal stocking density, the influence of basket depth had to be determined. Multiple papers found that stocking density limitations of other urchin species were influenced by basket depth (Christiansen and Siikavuopio, 2007; Daggett *et al.*, 2006; Devin, 2002; Siikavuopio, 2009; S. Siikavuopio *et al.*, 2007). Therefore, this study began by investigating if the basket depth would influence production of *T. gratilla* and why this might occur.
II. The number of urchins present in a tank will influence its water quality. Therefore, a stocking density expected to be used in commercial setting needed to be defined. This will additionally allow for estimation of *T. gratilla* production.
III. Measuring mass and diameter of large quantities of live urchins is necessary for commercial production. However, the stocking density and basket depth experiments revealed there was no appropriate or accurate method of doing this. To solve future bottlenecks that would occur, thereby reducing the feasibility of this system, a simple and highly accessible protocol was developed using computer vision.
IV. As TAN is likely the underlying exchange resource and primary limitation of production of both *Ulva* and urchins, it is necessary to predict the ammonia concentration in the urchin

effluent. Due to several factors that influence TAN concentration in an aquaculture system, this study investigates the capability of using regression models to predict it. To construct these regression models, a training data set needed to be created. This resulted in a trial during which many water samples were taken from urchin systems applied with various treatments, which were hypothesized to influence water quality. As many samples were taken, several other water quality parameters were observed. Therefore, this trial not only provided the training data required for the regression model but also offered an in-depth understanding of how urchin systems will influence the water quality on a whole. This provided novel insight, from which it was possible to make valuable management practice recommendations.

This project could be the final piece of the puzzle, putting together years of research into creating a new and sustainable aquaculture sector. This has the ability to contribute towards many of the United Nations' sustainable development goals such as:

1) No poverty (via job creation and GDP growth);
9) Industry innovation and infrastructure;
12) Responsible consumption and production;
13) Climate action; and
14) Life below water.

Chapter 2: Optimising basket depth and stocking density for *Tripneustes gratilla* production

Abstract

To assess the feasibility of *Tripneustes gratilla* culture, the quantity of individuals that can be sustainably produced in a given space needs to be determined. Prior to this analysis, it was necessary to investigate if and why the depth of the baskets containing *T. gratilla* influences their production, as it does for other urchin species. Contrary to suggestions from the literature, there was no clear evidence that deep baskets caused higher spine loss than shallow baskets. Nevertheless, these experiments provided important management recommendations; removing urchins from water for extended periods (> 5 s) or feeding them rigid feeds (such as kelp) results in significantly higher spine loss ($F_{(5, 12)} < 22.84$, $p < .0001$), which will reduce growth. Consumption was significantly higher in shallow baskets (15 cm deep) than deep baskets (30 cm deep) for both fresh *Ulva lacinulata* ($W = 38$, $p = 0.026$) and a formulated feed ($W = 76.5$, $p = 0.007$). This provides evidence that reduced consumption is the cause for reduced production observed for multiple urchin species in deeper culture systems. Therefore, baskets of approximately 15 cm deep are recommended to enhance the production of *T. gratilla*. The following stocking density trials were conducted in shallow baskets.

Two trials were conducted, where production parameters were compared between urchins held at three different stocking densities. The first was a three-month grow-out trial, where only fresh *Ulva lacinulata* was supplied and the objective was to maximise urchin size. The subsequent trial focused on maximising gonad production, where primarily a formulated feed was provided over two-months. While greater stocking density did significantly reduce the specific growth rates of average individual urchin mass in both trails ($p < 0.044$), there was no indication mortality or gonad size and quality was influenced by the densities used in this study. There was evidence that these contrasts in individual growth between densities were due to spine loss from negative behavioural interactions ($F_{2, 9}= 9.551$; $p = 0.005$). It is suggested that for the objectives of both the grow-out and gonad enhancement phases a stocking density of approximately 20% coverage (surface area of urchins' tests by surface area of basket) will be optimal.

2.1 Introduction

To determine the functional, environmental, and financial feasibility of a commercial, integrated multi-trophic aquaculture (IMTA) system for *Tripneustes gratilla* and *Ulva*, the ideal number of urchins contained within the basket or other containment units needs to be determined. The proportion of urchin biomass relative to the water volume or surface area of the system they are contained in (such as a basket), known as stocking density, will greatly influence the quality of the water within the system and its effluent. The design and management of a productive and balanced *T. gratilla -Ulva* IMTA system requires the ability to estimate and evaluate parameters, such as the total ammonia nitrogen (TAN) concentration, of the effluent. This requires knowing the principal input variables, such as stocking density. Furthermore, given that the primary motive of an aquaculture farm is financial, it is accepted that *T. gratilla-Ulva* farmers using IMTA systems will stock their tanks with an optimal density of urchins to achieve the highest yield and quality of urchin gonad. Despite being essential for the effective design and management of aquaculture systems and fundamental to financial forecasting, the optimal stocking density of *T. gratilla* has not been clarified. However, before an optimal stocking density for *T. gratilla* can be established, other aquaculture conditions, such as the design of the basket that could affect the maximum stocking densities also needs to be investigated. Depth of urchin basket has been observed to influence the production of urchins (Devin, 2002; Pearce, Daggett and Robinson, 2002; Daggett, Pearce and Robinson, 2006; Christiansen and Siikavuopio, 2007; Siikavuopio, Dale and Mortensen, 2007; Siikavuopio, 2009) and therefore will likely affect the assessment of urchin stocking density optimization. Therefore, prior to establishing an optimal stocking density for *T. gratilla*, it was necessary to determine the influence that basket depth has on stocking density. Baskets are placed in tanks or raceways and are used as a surface of attachment for urchins and for their management. While there are some other systems, most aquaculture facilities of benthic species use baskets. Thus, for the sake of applicability and simplicity the term “basket” is defined as the unit that the urchin is contained in and cannot leave, and therefore includes all forms of urchin holding units.

Optimising basket depth

Multiple studies have noted that deeper baskets result in substantially reduced urchin production (Christiansen and Siikavuopio, 2007; Daggett *et al.*, 2006; Devin, 2002; Pearce *et al.*, 2002; Siikavuopio, 2009; S. Siikavuopio *et al.*, 2007). One of the clearest examples of this effect is the contrasting mortality rates between two studies on *Strongylocentrotus droebachiensis*. Siikavuopio *et al.* (2007) observed high mortality (up to 80%) over 54 days with deeper baskets (69 cm), while Pearce *et al.* (2002) reported lower mortality (less than 6.5%) over 84 days with shallower baskets

(28 cm). Siikavuopio *et al.* (2009) denotes these two studies had nearly identical conditions (stocking density, flow rates and feeding regimes etc) with the only difference being basket depths, where the study with high mortalities had deeper baskets (69 cm) and the other study had shallower baskets (28 cm). Siikavuopio *et al.* (2009) goes on to hypothesize the reason for this contrast in mortality is due to a behavioural trait where urchins (of various species, including S. *droebachiensis* and *T. gratilla*) exhibit a preference for attaching to the upper portion of the sidewalls of baskets. They suggest the urchins must travel to the bottom of the basket to feed on pellets. In the process, they collide with one another, causing spine breakage. More injuries arise from collisions between urchins during vertical movements when animals are stocked at higher stocking densities (Siikavuopio *et al.*, 2007). All echinoderms are able to regenerate organs and appendages (Hyman, 1955), but spine regeneration effects the allocation of resources (Ebert, 1967; Edwards and Ebert, 1991). For example, there is an increased ratio of calcite production for spine growth compared to test (shell) growth after spines have been lost (Ebert, 1968). Haag *et al.* (2016) also found that spine loss is a significant aspect of the nutrient allocation in *Strongylocentrotus purpuratus* and stunts growth. Furthermore, there is evidence that increased spine loss causes a combination of reduced appetite (feed intake) and lower feed conversion efficiency, which results in a decreased gonadosomatic index (GSI) in S. *droebachiensis* (Siikavuopio, 2009). Essentially, the hypothesis is that deep baskets cause more collisions between urchins, resulting in spine loss which reduces urchin production. However, this had not been directly examined or proven prior to this study.

Other studies have also noted contrasts in urchin production due to basket depth. However, they have different theories on the mechanics behind it. The mortality rates of S. *droebachiensis* housed in small and shallow (15 cm) raceways had a considerably lower mortality rate (8%) over the experimental period compared to the mortality rate (24.3%) of urchins kept in large and deeper (25cm) raceways (Daggett *et al.*, 2006). While other variables could have played a role (specifically flow rates), Dagget *et al.* (2006) suggests the contrast in mortality is due to basket depth. Instead of proposing deeper baskets result in higher collisions between urchins (Siikavuopio, 2009), Dagget *et al.* (2006) hypothesizes there is a link between basket depth and urchin handling methods. The tanks containing urchins need to be cleaned and the standard practice is to remove the baskets containing the animals from the tanks during cleaning. This can be problematic because when urchins are removed from the water, they are unable to stay attached to the side walls for long and soon fall to the bottom of the baskets (Daggett *et al.*, 2006). This is harmful. Their suction cup tube feet tear off as they detach. The impact of falling onto the bottom of the basket can break spines and crack their tests (personal observation). The higher the fall, the more damage the animals are likely to suffer due to increased impact. During the Daggett *et al.* (2006) study, urchins in the small raceways remained submerged throughout the experiment. For the larger raceways, baskets were moved from one body of water to another during cleaning for the first four weeks of experimentation. During the moves, some urchins fell from the side walls and were possibly damaged. Daggett *et al.* (2006) suggests this is the cause for higher mortality and deeper baskets may result in lower production where they need to be removed from the water for cleaning.

Another theory suggests deeper tanks reduced feed accessibility. Devin (2002) reported that numerous *S. droebachiensis* farmers across the Northeast Atlantic have made similar observations regarding the inefficiency of deep, rectangular baskets. The aforementioned behavioural tendency of urchins congregating on the upper sides and corners of the tanks results in not only inefficient use of space but also limits access to feed. Like most urchin species, *Tripneustes gratilla* are known to have low mobility. In the wild, they have been observed to travel on average only 1 m per day in a random direction (Koike *et al.*, 1987a; Stimson *et al.*, 2007). Therefore, it is suggested that if food is not delivered directly to them, consumption will be reduced (Devin 2002). When feed settles on the bottom of the enclosure and urchins are clustered at the top, it is less likely they will consume the feed. If they do, it requires them to move, which expends energy and resources that would otherwise be allocated to growth. This suggests that shallow and narrow tanks should result in greater feed accessibility, which leads to higher consumption and thus, improved production. Again, this had also not been explicitly tested.

While these theories imply superiority of shallow baskets for urchin production, deeper baskets have practical benefits. Where cost of coastal land often inhibits this industry (Bolton *et al.*, 2009), most commercial, land-based aquaculture systems use deeper tanks because they support a higher stocking density of animals per surface area of the farm itself. Furthermore, deeper systems have less relative surface area for heat transfer and thus experience reduced temperature fluctuations, thereby saving heating and cooling costs where applicable. In addition, larger volumes of water may also reduce fluctuations in water chemistry because of the increased buffering effect. Shallow systems that are stacked in deep tanks can offer similar benefits as detailed above (Devin, 2002; Grosjean *et al.*, 1998.). They are, however, considerably more demanding in terms of design, management and expense. It is important to acknowledge that maximising stocking density is not the sole objective of an urchin farm. Profit, sustainability and animal ethics are other factors that need to be considered.

No research had been conducted into *T. gratilla* basket depths, although, as has been observed in other species previously mentioned, *T. gratilla* also favours the upper portion of baskets (personal observation), which suggests that basket depth production limitations seen in other species might apply. Given that the pellets used by Siikavuopio *et al.*, (2007) sunk to the bottom, it is necessary to determine the combined effects of basket depth and feed type. If the feed does not settle on the bottom and can be captured and consumed from the sidewall (such as fresh *Ulva*), the depth may not have an influence. Long-term maintenance of urchins requires them to be removed from the water while in their baskets. To ensure this study could be applied practically, the effects that different basket depths have on urchin spine loss when removed from the water also needed to be investigated. If the system depth affects urchin production and the incorrect depth is used when determining an optimal stocking density, it could result in a lower production rate. This would affect the feasibility of the industry. If the depth is optimised initially, it will provide an accurate foundation on which to establish production parameters. Furthermore, these experiments can provide evidence to prove or disprove existing theories, and results can be applied to system design for other urchin species that display similar behaviour.

Optimising stocking density

Once the existence and/or extent of the effect of basket depth is determined, the stocking density of *Tripneustes gratilla* can be investigated. For a given animal aquaculture system, the two primary factors that are generally considered to limit stocking density are water quality and behavioural interactions (MacIntyre *et al.*, 2008). A study was conducted where various stocking densities of *T. gratilla* were subjected to different water exchange rates, which provided valuable insight into how this species changes water chemistry and how this affects their somatic and reproductive growth (Mos *et al.*, 2012). Further analysis into the influence of stocking density on water quality is provided in Chapter 4 of this thesis.

The water quality of an aquaculture system can be improved through simple measures, such as increasing the water exchange (turnover) rate. Yet, even if water quantity parameters remain below toxic levels, it is not possible to increase stocking density *ad infinitum*. At some point, the number of urchins in the system will reach capacity, whereafter adding more will result in negative interaction between them. Examples of negative behavioural interactions between urchins include the aforementioned collisions that result in spine loss (Siikavuopio *et al.*, 2007), feed competition/accessibility (Richardson *et al.*, 2011) and cannibalism (Le Gault and Hunt, 2016;

Richardson *et al.*, 2011; Sonnenholzner *et al.*, 2011). The frequency and severity of these interactions are hypothesized to increase proportionally with stocking density. This is because reduced surface area increases encounters between individuals and may cause stress. While optimal stocking densities would likely be the result of a combination and/or interaction between water quality and behavioural interactions, it was important to examine these limitations individually to isolate their effects.

A further complication with stocking density analysis of urchins is that there is no standard measurement. Many papers report the number or mass of urchins per metre squared or cubic metre. This is problematic because it is often unclear whether the study is referring to the area the holding system occupies on the farm or the actual surface area within the holding units. Reporting density only as a factor of farm area is not meaningful because, as previously described, urchins prefer to occupy the side walls of the enclosure and not the bottom. Therefore, the depth has a significant effect on the stocking density (Daggett *et al.*, 2006; Devin, 2002; S. Siikavuopio *et al.*, 2007). For the same reason, it is more valid to report stocking density as a factor of surface area and not only volume. It is also necessary, however, to report the water exchange rate.

Reporting the number of urchins and not clearly stating the sizes of the individuals is also limited in its usefulness. A more appropriate metric used to describe stocking density in this study is $kg.m^{-2}$, where the area refers to the surface area inside the holding system occupied by the urchins. This metric though, is not ideal because it cannot be applied evenly across various size classes of urchins. For example, if a stocking density of 8 $kg.m^{-2}$ is found to be a threshold limit for urchins of 100 mm diameter, this will not apply to urchins of 10 mm. This is because the mass-to-surface area ratio of urchins is exponential and not linear (Balisco, 2015). When compared to adult urchins, juveniles occupy a much larger space relative to their mass. Therefore, if a basket was stocked with juveniles to 8 $kg.m^{-2}$, there would be limited space between urchins, which would likely result in reduced production. For this reason, this study primarily utilizes the more relevant and meaningful percentage cover as stocking density metric (James *et al.*, 2017). This is the surface area all the urchins' tests (not including spines) in a basket occupied as a proportion of the total available surface area. This is likely to be more relatable across urchin size classes. The applicability of this can be demonstrated using the previous example. If urchins of 100mm in diameter were stocked at 8 $kg.m^{-2}$ in a basket with an internal surface area of 0.4 m^2, the 23 individuals required to achieve this density would occupy approximately 18.21% of the basket's surface area. Yet, if urchins of 10 mm were also stocked in the same size basket, the 4 363 individuals required to achieve 8 $kg.m^{-2}$

would occupy 85.67% of the basket. This metric may be more relatable between urchins in different size classes and different enclosures. Therefore, it has also been used in this study to compare papers, which report performance of *T. gratilla* at various stocking densities (Table 2.1).

Table 2.1. The most relevant data extracted or calculated from papers which provide insight into stocking density of *Tripneustes gratilla*. Some data are approximate as they were not explicitly provided and therefore is read off graphs and/or deduced by calculation. To determine mass from diameter or vice versa, the exponential relationship from Balisco (2015) was applied. GSI (gonadosomatic index) is the urchin's gonad mass relative to total wet mass of the urchin (Equation 2.5). SGR (specific growth rate) is rate of urchin growth relative to time (Equation 2.6).

Reference	**Water exchange rate**	**Feed**	**Initial average size**	**Experiment period**	**End average size**	**Initial stocking density**		**End stocking density**		**SGR**	**GSI**	**Survival**
	Exchanges per hour		Test diameter (mm)	Days	Test diameter (mm)	Kg/m^2	% cover	Kg/m^2	% cover	Test diameter (mm)	%	%
Juinio-Menez *et al.*, 2008	NA [d]	Sargassum	26.77	390	74.94	0.56	3.06	7.67	21.21	0.35	5.86	92.31
					72.45	1.50	8.40	15.47	43.74	0.34	3.68	67.91
Asia *et al.*, 2009	ND [e]	Sargassum	45.5	120	93.8	1.92	8.19	4.35	13.57	0.11	5.77	ND
Mos *et al.*, 2012 (juvenile)	3	Sargassum	21.7	70	67.66	0.15	1.37	3.72	13.32	1.62	NA	>90
						0.30	2.74	5.93	21.65	1.48	NA	>90
						0.45	4.11	7.78	23.60	1.25	NA	>90
Mos *et al.*, 2012 (adult)[b]	3	Sargassum	74	42	97	0.81	2.93	1.67	4.84	0.59	5.2	100
						2.44	8.77	4.21	13.78	0.54	5.1	100
						4.06	15.19	6.91	21.78	0.43	3.4	100
Manuel *et al.*, 2013	NA [d]	Sargassum	38.67	150	86.69	1.74	8.39	10.77	42.16	0.54	9.79	ND
Cyrus *et al.*, 2013	ND [e]	Fresh *Ulva* then artificial feed	33	224	78	0.11	0.57	1.32	3.19	0.38	17.5	100
Shpigel 2018[c]	2.5	Pellets	24.98	251	44.44	0.3	2.09	2.09	6.62	0.22	14.4	78
		U. lactuca			73.28			7.73	18.00	0.43	13.7	88

[a] Mass = 0.733 x (Diameter)$^{2.673}$.

[b] For the sake of this comparison focusing on behavioural interactions, only the data points of the high exchange rate (three per hour) were considered.

[c] Lower survival was due to a system malfunction.

[d] Not applicable as the experiments were conducted in the ocean.

[e] No data provided in the paper.

There is a considerable amount of literature investigating the effects of stocking density on other urchin species (Cárcamo, 2015; James *et al.*, 2017; Qi *et al.*, 2016; S. Siikavuopio *et al.*, 2007; Siikavuopio and James, 2011; Warren-Myers *et al.*, 2020). Unfortunately, it cannot be used for direct comparison for *T. gratilla* as it has been shown that different urchin species have different stocking density requirements (Suckling *et al.*, 2020). No study has been conducted to specifically investigate how behavioural interactions limit stocking density. There is though, literature providing some insight into appropriate stocking densities of *T. gratilla* which is used by this study to determine which stocking density values to test. These comparisons are not absolute because of the presence of other factors. These are primarily water quality and temperature, which would have fluctuated within most of these experiments and may have had confounding effects. A stocking density experiment was conducted in a series of sea cages over a 390-day period (Juinio-Meñez *et al.*, 2008). There was no significant difference in the specific growth rate (SGR) of the test diameter in the lower density. Nevertheless, there was significantly higher gonadosomatic index (GSI) and survivorship (Table 2.1). This implies that the higher stocking density (ending at approximately 15.47 kg.m^{-2} or 43.74% cover) may be too high. The lower stocking density treatment, ending at 7.67 kg.m^{-2} or 21.21%, resulted in an average GSI of approximately 5.86%, which may be considered low compared to other studies but is nearly twice that of the high stocking density treatment. This value is similar to that from a study by Asia *et al.* (2009) in which fresh *Sargassum sp.* was also fed. The animals were, however, held at a lower stocking density of approximately 4.35 kg.m^{-2} or 13.57% cover (Table 2.1), suggesting that a stocking density around 20% will not affect GSI. Contrary to this, Manuel *et al.*, (2013), who used similar culture methods to Juinio-Meñez *et al.* (2008), reached similarly high stocking densities but found a considerably higher GSI and SGR (Table 2.1). The reason for this is not clear but survival and other specifics were not reported in this study. Therefore, the deduced end stocking density of around 10 kg.m^{-2} or 40% cover (Table 2.1) may be a threshold.

The stocking density analyses of Mos *et al.* (2012) provides valuable insight as it was conducted for both juvenile and adult urchins at various densities and water exchange rates. For the sake of this comparison, that is, focusing on behavioural interactions, only the data points of the high exchange rate (three per hour) were considered (Table 2.1). Juveniles and adults had significantly reduced SGR at higher stocking densities, but there was no evidence that density affected mortality. While this study attributes most of the differences in performance between treatments to pH and CO_2 and its influence on the availability of carbonate ions, it did find that the higher stocking densities of juveniles had a greater variance of size between individuals in a tank. This implied that certain individuals were outcompeting others for food, therefore growing larger. There was a correlation between reduced production (in terms of SGR and GSI) with increasing stocking density. On the contrary, for the adult urchins, there was considerably less difference in performance between the low and medium density than between medium and high density. This implied that the higher density of 6.91 kg.m^{-2} or 21.78% cover may result in greater negative behavioural interactions, which reduce production, although this may have also been the effect of different pH or pCO_2 concentrations.

A feed experiment by Cyrus *et al.* (2013) investigated the effects on *T. gratilla* production with the feeding regime of 20 weeks of fresh *Ulva lacinulata* (as *Ulva rigida*), followed by 12 weeks of artificial feed with 20% *Ulva* inclusion. This was done at low densities and provided a benchmark for urchin growth over a full lifecycle, with an SGR of 0.38 across all treatments and some of the highest GSI recorded. A similar study of *Tripneustes gratilla elatensis* used feed formulated to the same chemical composition as the previously mentioned study and found considerably lower survival, SGR and GSI (Shpigel *et al.*, 2018). The lower survival was due to a system malfunction. The reduced GSI and SGR were attributed to the differences in the feed's ingredients and stability or differences in sub-species (Shpigel *et al.*, 2018). These differences may have also been the result of less frequent feeding or perhaps higher stocking densities in Shpigel *et al*. (2018). The GSI across all treatments of Shpigel *et al.* (2018) was still notably higher than the average seen in the literature. This demonstrated high GSI can be achieved in higher stocking densities if the correct feed is supplied.

The aforementioned seven studies vary greatly in results regarding stocking densities and, while no clear picture is demonstrated, it is suggested that 15.47 kg.m^{-2} or 43.74% cover is too high resulting in low survival (Juinio-Meñez *et al.*, 2008). There are clear cases where urchins held at stocking densities of approximately 8 kg.m^{-2} or 23% cover had similar performance (Shpigel *et al.*, 2018) to the maximum performance observed (Cyrus, 2013). In most cases, the water quality did not appear to be at levels of concern, suggesting that a stocking density where the behavioural interactions begin to limit production is near 8 kg.m^{-2} or 23% cover.

This study aimed to further clarify the effects of stocking density on negative behavioural interactions and its limitations on *T. gratilla* production. To achieve this in a means relevant to commercial production, experimental systems should be as representative of commercial systems as possible. Ideally, the sizes and dimensions of the culture systems should reflect those anticipated in commercial settings. Furthermore, the feeding regimes must mirror those expected in commercial operations. It was suggested that there would be two separate growth phases once juvenile *T. gratilla* shifted from their benthic biofilm diet. The first phase would be the grow-out phase where the objective was to maximise somatic growth, specifically test diameter and height. There is evidence that the feeding of fresh *Ulva* is most efficient for this phase (Cyrus *et al.*, 2015). The objective of the second growth phase is gonad enhancement. Here, a mostly high protein, artificial feed with a 20% *Ulva* inclusion and a small portion of fresh *Ulva* would be optimal (Cyrus *et al.*, 2015). The different feed types and objectives may be influenced by behavioural interactions and therefore different optimal stocking densities would apply. For this reason, separate stocking density experiments needed to be conducted. Furthermore, experiments investigating specific behavioural interactions that might influence production at different stocking densities were undertaken.

Aims and objectives

The initial aim of this study was to investigate why basket height influences the production of *Tripneustes gratilla* based on theories presented in the literature. To achieve this, the four objectives were to: determine the relationship between basket height and spine loss, investigate the effect of removing baskets of varying height from the culture system on spine loss, find whether consumption is influenced by basket height and examine if basket height can affect the mortality rate. Once a recommended basket height was established, the second aim was to determine the optimal stocking density of *T. gratilla* at the recommended basket height, at both the grow-out (*Ulva*-fed) and gonad-enhancement (pellet-fed) phases of the urchin production cycle. Lastly, this study investigates possible reasons for potentially reduced production at higher stocking densities.

2.2 Methods

2.2.1 System set-up

The experiments were conducted at the Department of Forestry, Fisheries and Environment's (DFFE) Marine Research Aquarium in Sea Point, Cape Town, South Africa. All trials were conducted in three 1 386 l plastic tanks (140 x 110 x 90 cm, length × width × height). Each tank was supplied with 75 µM filtered seawater. It was recirculated through a sand and biofilter with a daily replacement rate of 84% and maintained at a constant temperature of 25°C using a heat pump. This temperature of 25°C was used as it was the nearest value to the optimal temperature range of 26-28°C for *T. gratilla* (Mos *et al.*, 2012) that could be consistently achieved at the facility where this study was conducted. Each tank had an hourly water exchange rate of 0.5 (full water exchange

- turnovers per hour). The incoming water entered each tank parallel to the water surface at a depth of *ca.* 15 cm and at a high velocity (*ca.* 700 l/hr) to ensure that there was a high level of circulation in each tank. All tanks were constantly aerated using an air stone to further promote circulation/mixing of water. Urchins were housed in baskets of varying dimensions (details provided below) within each tank and were made from HDPE plastic mesh (3 mm mesh size) supported by PVC conduit. The bottom of each tank was siphoned clean of debris and faecal matter once every second day to ensure optimal water quality.

2.2.2 Feeds

Ulva lacinulata was sourced from the Buffeljags abalone farm in the Western Cape of South Africa, where it is cultivated in raceways. The nutrient analysis (Table 2.2) was conducted at a private laboratory, Microchem Lab Services (Pty) Ltd, using protocols described by AOAC International (2002). The crude protein content was determined by multiplying the nitrogen content by a factor of 5.45 (Angell *et al.*, 2017). The artificial feed used in this experiment was the same formula as described in Cyrus *et al.* (2015) with 20% dried *Ulva* inclusion in extruded chips with approximate dimensions of 40 x 10 x 5 mm, L x W x H . Nutrient analyses (Table 2) were conducted by the same laboratory using the same methods as described above. Stability of the formulated feed was determined by recording the percentage dry matter lost after the feed was submerged in the same tank and the same type of basket the urchins were cultured in (thus with the same conditions) for 24 h.

Table 2.2 The nutrient content of the feeds used (per dry weight).

Feed	***Ulva lacinulata***	***Artificial feed***
Protein (%)	20.71	32.30
Fat (%)	1.36	4.22
Moisture (%)	15.30	8.30
Ash (%)	27.34	13.50
Calcium (%)	0.34	1.26
Magnesium (%)	2.61	< 0.01
Gross energy (MJ/kg)	No Data	12.26

The kelp (*Ecklonia maxima*) used in this study was wild kelp collected directly in front of the Marine Research Aquarium. Only the fronds, cleaned of epiphytes and other debris, were used. A nutrient analysis was not conducted for the kelp as it was only used for a single experiment that did not measure growth rates.

2.2.3 Basket depth experimental set-up

Each of the three large plastic tanks described above was equipped with six baskets, including three deep baskets and three shallow baskets (Fig. 2.1) that were suspended in the tanks. The arrangement of baskets in this trial, and all those to follow, was randomised to reduce positional bias.

Fig. 2.1. Photograph of deep (40 cm × 15 cm × 30.9 cm, Length × Width × Height) and shallow baskets (40 cm × 40 cm × 15 cm) used for the basket height experiments. The dimensions of the baskets differ but the surface area within the baskets is the same.

Both the shallow and deep baskets had an available internal surface area (ISA) of 0.4 m^2 (Equation 2.1). To maintain a constant available area for the urchins while varying the depth of the baskets, the width was adjusted accordingly. This was the only approach viable and there are no conceivable ways varying widths of the baskets would influence the urchins. The basket widths were always greater than twice the maximum height of the urchins used in this study, meaning there was sufficient space for the urchins to move past each other when positioned oppositely within the enclosure in both baskets. The calculation of ISA will vary depending on the design of the urchin enclosure, where all surfaces that urchins can attach to should be consider (such as baffles and lids), however the follow calculation was used for the baskets used throughout this study (Equation 2.1).

$$\mathrm{ISA} = \mathrm{lw} + 2(\mathrm{hw}) + 2(\mathrm{hl}) \tag{2.1}$$

Where:

ISA = internal surface area

l = Length of basket

h = Height of basket

w = Width of basket

Tripneustes gratilla used in in this experiment were produced and reared from larvae at this facility. For four months prior to this experiment, urchins were held at similar stocking densities, in baskets of identical design and fed a mixture of *Ulva* and kelp. At the beginning of the experiments detailed below, each basket was stocked with 22 individual sea urchins with an average mass of 86.36 g and test diameter of 65.71 mm. This resulted in a biomass of 1.9±0.03 kg (Mean ± SD) that equates to a stocking density of approximately 4.75 kg.m^2 in each basket (Equation 2.2).

$$\text{Stocking density by mass } = \frac{\text{Total urchin mass}}{\text{ISA}} \tag{2.2}$$

All the urchins were haphazardly placed in their respective baskets and left for five days without feed to acclimatize before the experimentation began.

2.2.4 Effects of basket depth and feed type on spine loss

A complete 2×3 factorial experimental design with two-treatment levels, basket depth and food type, was used for this experiment. The three different types of feed tested included: formulated feed (pellets) containing 20% dried *Ulva lacinulata*, as described in Cyrus (2015a); fresh *Ulva lacinulata*; and fresh kelp (*Ecklonia maxima*). The 2×3 factorial design resulted in six treatments: (1) deep baskets fed with *Ulva*, (2) deep baskets fed with pellets, (3) deep baskets fed with kelp, (4) shallow baskets fed with *Ulva*, (5) shallow baskets fed with pellets, and (6) shallow baskets fed with kelp. There was one treatment per tank (experimental unit), thus three replications per treatment. As limited resources (space and tanks) only allowed three replications per treatment and variance was expected to be high, the entire experiment was repeated on three separate occasions (13/05/2019, 22/05/2019, 30/05/2019). Each experiment was treated in an identical fashion and ran for three days. Prior to the start of each experiment, all urchins were starved for five days to stimulate higher consumption and increase the likelihood that the effect could be detected. To collect the lost spines, 200 µm mesh bags were placed around each of the baskets before the urchins were fed their respective diets. All excess pellets and kelp were removed daily (as they decompose, unlike *Ulva* that was subsequently not removed) and the urchins were fed ad libitum. After 72 hours, the mesh bags were carefully removed, and the faeces were separated from the broken spines by filtration and panning. The spines were dried to a constant weight in an oven at 60°C and weighed to the nearest 0.001 g.

2.2.5 Effects of basket removal and depth on spine loss

The experiment began on the 03/06/2019 and ran for a period for seven days using the same system and *Tripneustes gratilla* as described in section 2.2.1. In each of the three tanks, there were three shallow baskets and three deep baskets, as previously described. During the experiment, urchins were not fed, and 200 µm mesh bags were placed around each of the baskets to capture spines. During the experiment, one set of deep and shallow baskets from each tank were not removed, whereas the second set of baskets was removed for five seconds once every day and the third set of baskets was removed for five minutes once every day. At the end of the week, the mesh bags around the baskets were removed and spines were separated from the other material in the mesh bags, before drying spines to a constant weight in an oven at 60°C overnight and weighing to the nearest 0.001 g.

2.2.6 Effects of basket depth on consumption

The experimental set up was as described in 2.2.1, with the exception that the mesh bags were not used as spines or faeces did not need to be collected. Treatments were also similar, with the exception that kelp was not used. Prior to the initiation of the experiment, urchins were starved for five days. The experiment started on the 15/05/2019 when each basket received a known mass of allocated feed. The mass of fresh *Ulva* was recorded after rotating the *Ulva* in a salad spinner 20 times before being weighed, which brought it to a constant weight (MJ Brand, unpublished). The salad spinner has a volume of *ca.* 3 l and the mass of *Ulva* spun at a time would be *ca.* 0.5 kg. All baskets were provided with approximately 120 g of fresh *Ulva* or 25 g of pellets. This quantity was administered to ensure that surplus food always remained after 24 hours, and that food was not a limiting factor in the experiment. The feed was supplied daily at midday and removed the following day at the same time. The *Ulva* was removed both by hand and siphon and then spun and weighed as described above. The uneaten pellets were carefully siphoned through a 200 µm filter. The pellets were placed in an oven at 60°C until weight was constant. The samples were then weighed to the nearest 0.001 g. To account for the changes in feed mass not caused by the urchins, it was necessary to conduct a feed stability test. This was done by adding a portion of each feed type in a basket without urchins and measuring the weight of the feed before and after being in the water for 24 hours. The process of determining the difference in mass of the feed over a 24-hour period was repeated six times (over six days) for the pellet-fed baskets and four times for the *Ulva*-fed baskets. The values of these replicates were averaged for each basket to meet the assumption of independence. Rate of consumption was calculated via Equation 2.3:

$$\mathrm{IR} = \mathrm{F_p} - \mathrm{F_R} \tag{2.3}$$

Where:

IR = ingestion rate

F_p = weight of feed provided

F_R = weight of feed not eaten – weight loss/gain in control.

2.2.7 Effects of stocking density on the grow-out phase

This trial compared the growth and gonad development of *Tripneustes gratilla* held at various stocking density while they were fed fresh *Ulva lacinulata* ad libitum over a three-month period. Baskets were stocked with adult *T. gratilla* that had been fed the same mixed diet of U. *lacinulata* and *Ecklonia maxima* and housed at the same stocking density (6 kg. m^{-2}) for one month and then starved for five days before the experiment began on the 08/10/2019. The baskets for this trial measured 40 × 40 × 15 (L×W×D, ISA = 0.4 m^2). These dimensions were chosen based on the results of the basket dimension trials described above and because they needed to be as near to commercial scale as logistics would allow. Diameter, height and mass of all urchins from the holding tank were recorded (using vernier callipers and a scale) and only animals with a test diameter of 62-70 mm were used in the experiment. The average mass of the urchins was 109.73 g. Each of the three baskets in each tank was stocked at a different density, providing three treatments and three replicates across the three tanks. The initial stocking densities were of 4, 6 and 8 kg.m^{-2} (Equation 2.2) or 13, 19 and 24% coverage of the available basket surface area (Equation 2.4) made up of 15, 22 and 29 individuals, respectively.

$$\text{Percent coverage} = \frac{\sum_{i=0}^{n} \pi r^2}{\mathrm{ISA}} \tag{2.4}$$

Where:

$\sum_{i=0}^{n} \pi r^2$ = the total area occupied by all urchins in the basket

ISA = internal surface area of basket

At the end of the three-month experiment, diameter, height and mass were determined using vernier callipers and a scale for each urchin from every basket. Two individuals from each basket were then haphazardly selected, dissected and their gonads weighed. A hand-held spectrocolorimeter (Lovibond LC100) was used to record the lightness (L*), redness (a*) and yellowness (b*) of the gonads. Three repeated measurements of these light values were taken of each gonad, which were then averaged. To ascertain initial colour values for the gonads, 10 urchins of the same size class and cohort were dissected at the beginning of the experiment prior to treatment. Histological analysis of gonads was conducted to determine whether the reproductive phase of the urchins was affected by stocking density. To do this, a single gonad from each urchin was placed into Davidson's Fixative (three-parts 95% ethyl alcohol, two-parts 100% formalin, one-part glycerol, one-part glacial acetic acid and three-parts distilled water) and fixed for 48 h, before being transferred into 70% ethanol and processed for routine hematoxylin and eosin histology (Bucke, 1989). The reproductive phase (stage of gonad maturity) of each gonad was then determined using methods described by Cyrus *et al.* (2013).

2.2.8 Effect of stocking density on the gonad-enhancement phase

This trial compared the growth, specifically reproductive growth, of *Tripneustes gratilla* held at various stocking densities, and fed formulated feed with 20% dried *Ulva lacinulata*, over a two-month period. The initial intention was to run this experiment as a continuation of the previous experiment, but this was interrupted by COVID and the ensuing lockdown. Consequently, the urchins from the above experiment were mixed and maintained for a period of three-months at a stocking density of ca. 6 $kg.m^{-2}$ and fed ad libitum on a diet of *Ecklonia maxima*. Once access to the facility was regained, the experiment began on 20/07/2020.

For this experiment, an additional basket was included for each treatment in each tank to increase the sample size and help reduce unexplained variability. Consequently, the baskets used were slightly smaller than those used previously and were 25 x 40 x 15 cm (L×W×D, ISA = 0. 295 m^2). The water flow rate in each tank was increased to 0.65 tank exchanges per hour to counter the additional dissolved nitrogen leeched from the formulated feed. Urchins with diameters between 68 and 86 mm and an average mass of 170.1 g were randomly selected for each treatment and a sub-sample (n = 10) was dissected to get initial GSI values (Equation 2.5).

$$\mathrm{GSI} = \frac{M_G}{M_U} * 100$$

(2.5)

Where:

GSI = gonad stomatic index

M_U = urchin mass

M_G = gonad mass

The stocking densities were similar to those from the previous experiment; 4 , 6 and 8 kg.m^{-2} or 10.49, 15.24 and 20.75% cover, holding 7, 10 and 14 individuals, respectively. The experiment also included a treatment where urchins were held individually so that they could not physically interact with one another. This was done by segregating the baskets into units measuring 13.3 x 20 x 15 cm (L×W×D) with the resulting stocking densities ranging from 1.04 to 1.9 kg.m^{-2} (1.46 to 2.13% cover). All treatments were fed the formulated feed (pellets) with 20% dried *Ulva lacinulata* at a rate of approximately 1.5% of the total urchin mass in each basket. Animals were fed on a Tuesday, Thursday and Sunday. Based on preliminary experiments (unpublished), the 1.5% feed ration was deemed optimal, since all feed was consumed within 24 hours, thereby avoided surplus feed leaching into the water column and adversely affecting water quality. On Fridays, the urchins were fed fresh *Ulva* at a rate of 6% of total body mass in each basket. All *Ulva* would be consumed by the following week. *Ulva* was fed once a week to improve the colour of the gonads (Cyrus *et al.* 2015), and to improve the quality of the water. At the end of the two-month experimental period the body mass, test diameter and height for each individual was assessed. Five individuals from each basket were randomly selected and dissected for gonad analysis (GSI and histology), as previously described.

2.2.9 The effect of stocking density on urchin behaviour (consumption, faecal production and spine loss)

To investigate the why stocking density effects urchin production within the same water quality urchin behaviours were quantified, specifically consumption (measured via both feed reduction and faeces production) and spine loss. This used an identical experimental design as described above for the gonad enhancement experiment. This experiment started on 29/02/2020. There were six replicates for each of the stocking densities (4, 6 and 8 kg.m^{-2}) as well as three baskets, one in each tank, each containing six individually housed *Tripneustes gratilla*. A 200 µm mesh bag was placed around each basket and animals were fed their respective feeds. All urchins were fed with 100, 150 and 200 g (±0.05 g) of fresh *Ulva lacinulata* (after being spun in a salad spinner 20 times, to achieve constant weight) for the 4, 6 and 8 kg.m^{-2} stocking density treatment groups respectively. The individually housed animals were each provided with 10 g of *Ulva*. Feed amounts were determined from preliminary experiments to ensure that there was always feed available during the feeding periods of this experiment. The feed remained in the baskets for 24 hours, after which uneaten food was removed by siphoning it out with care to minimise spine loss. The remaining feed was weighed and subtracted from the starting mass to determine consumption (Equation 2.3). The stability of the feed was factored in, as described previously. The following day, the mesh bags were carefully removed without taking the baskets out of the water. The spines and faecal matter were removed from the bags and separated using panning. The matter was then dried separately over 48 hours to a constant weight (at 60°C) and weighed. The mesh bags were reattached to the baskets. Treatments were fed the respective quantities and this experiment was repeated twice more (starting on the 04/03/2020 and 08/03/2020) in the same fashion. The experiment was not repeated with the formulated feed because a preliminary trial revealed the water flow was reduced by the mesh bags, which meant the elevated toxic waste products (such as ammonium) negatively affect the urchins and skew the experiment.

2.2.10 Water quality monitoring

Water quality was assessed during both stocking density trials to support the assumption that only behavioural interactions and not water quality affected growth, behaviour and gonad development across treatments. Oxygen concentration and pH were assessed between 9 and 10am once at the end of each month during these trials, using a probe (DTK2017-SD, Lovibond Tintometer GmbH) which was calibrated before use each time. Samples were taken from inside each basket and from the inflowing water. During the gonad enhancement trial when pellets were fed, the ammonium was also determined in both sampling times an hour after the pellets were fed, which is when nitrogen levels were likely to be at a maximum (Piedecausa *et al.*, 2010). The ammonia analysis was determined using Palintest® ammonium test kit (Palintest®, Gateshead, UK).

2.2.11 Statistics

The R statistical computing environment (R Development Core Team, 2017) was used alongside Microsoft Excel for data organization. Through discussions concerning the experimental designs, this study ensured the assumptions of independence and non-selectivity were met.

To explore the effect of basket height, feed type, and basket removal time on spine loss, this study analysed average daily spine loss data. For processing multiple treatment factors and facilitating pairwise comparisons, this study applied a linear regression model with least-squares means. Least-squares means predict from linear models, summarizing factor effects and testing linear contrasts in data analysis (Lenth, 2016). The model's assumptions were duly met (Appendix A and B). Pairwise T-tests were used for pairwise comparisons. Linear models were chosen over ANOVAs due to their superior ability to detect interactions.

For investigating basket height's influence on pellet and *Ulva* consumption, separate analyses were carried out for each, relying on average daily consumption over the experimental period. These datasets fulfilled assumptions of independence, normality, and homoscedasticity (Appendix C). Considering limited replication and the potential for a type II error, a Mann-Whitney U test was chosen over a one-tailed two-sample t-test.

To analyse the effects of basket height on mortality, a general linear model of the Poisson family was used to identify a factor that could correlate with mortality. Factors modelled included basket removals, feed type, blocks (tanks) and their interactions.

The stocking density experiments were randomised complete block designs (RCBD), where tanks were treated as blocks. The required assumptions were tested (Appendix D and E) to allow for the application of two-way analysis of variance (ANOVA) and Tukey tests. The specific growth rate (SGR, Equation 2.6) of mass and height was log-transformed to be normally distributed. The initial stocking density experiment, the *Ulva*-fed grow out trial, is a RCBD with a single replicate (basket) per block (tank) which means the assumption of additivity is required (Wilk, 1955). Boxplots with indication of blocks demonstrated that GSI, colour a* and colour b* were non-additive (Supplementary material E), therefore statistical tests were not applied to these variables as there are currently no alternative tests available. The visualisation of the data provided sufficient insight for these parameters. The remaining parameters all met these assumptions without transformation.

$$SGR = 100 \times \ln (L_T/L_0)/t \tag{6}$$

Where:

SGR = specific growth rate

L_0=initial length/mass

L_T=final length/mass

t = days of culture

Change in coefficient of variation (CV) was also calculated to assess the presence of adverse behavioural interactions between *T. gratilla* held at various stocking densities (Qi *et al.*, 2016). This was done by subtracting the CV recorded between individual urchin mass within each basket at the end of the experimental period from the CV from the beginning of the experiment. To investigate the relationship between faecal production and consumption, Pearson's product-moment correlation was applied.

To assess whether the water quality between treatments significantly differed, a generalized linear model was constructed that encompassed additional factors (blocks, dates of sample collection, and feed type) potentially influencing water quality. The validation of assumptions involved assessing the spread of residuals, quantile-quantile (Q-Q) plots, and a frequency graph (Appendix F), all of which indicated the assumptions were satisfied.

2.3 Results

2.3.1 Effects of basket depth and feed type on spine loss

Basket height had no significant main effect on spine loss ($R^2 = 0.925$, $F_{(5, 12)} = 22.84$, $p = 0.842$). Regardless of height, kelp resulted in significantly higher spine loss than *Ulva* ($R^2 = 0.925$, $F_{(5, 12)} = 22.84$, $p < .0001$) and pellets ($R^2 = 0.925$, $F_{(5, 12)} = 22.84$, $p < 0.001$). There was no significant difference in spine loss between urchins fed pellets and *Ulva* ($R^2 = 0.925$, $F_{(5, 12)} = 22.84$, $p = 0.995$) when maintained in deep or shallow baskets. None of the interactions within this analysis where found to be significant (Fig. 2.3, $R^2 = 0.925$, $F_{(5, 12)} = 22.84$, $p > 0.407$).

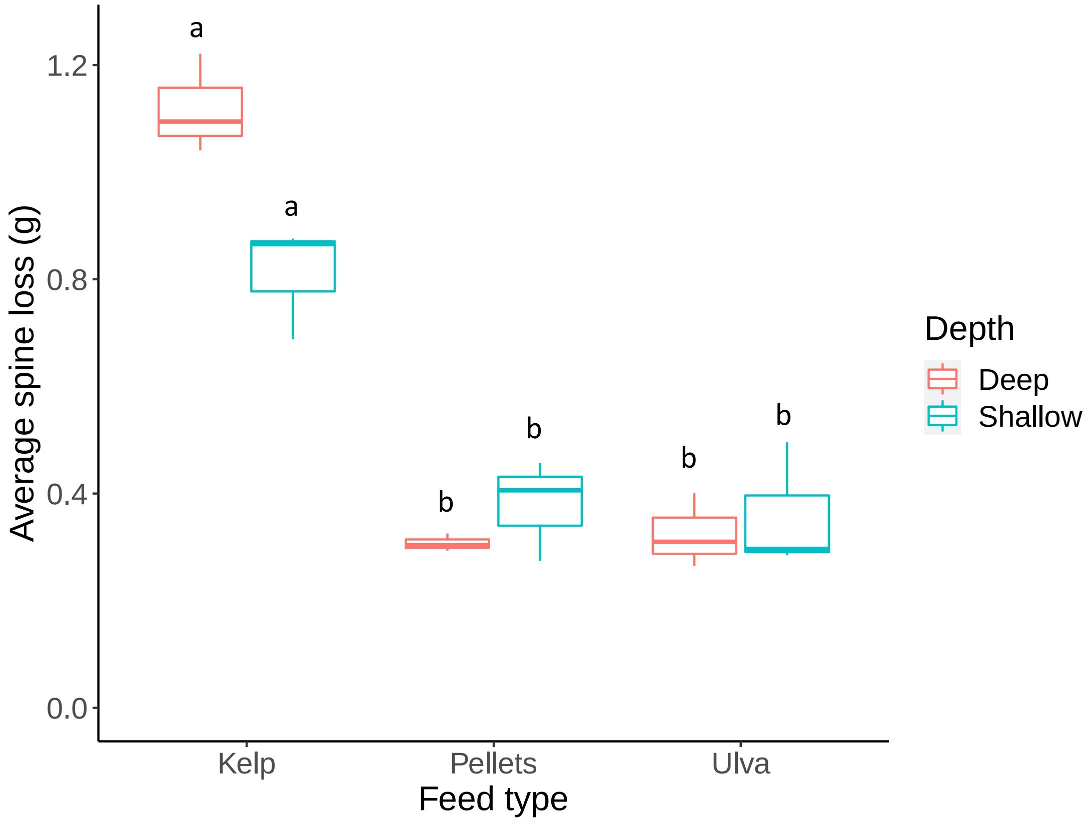


Fig. 2.3. The daily spine loss of *Tripneustes gratilla* over the experimental period, when fed different feed types and maintained in the deep (30 cm) or shallow (15 cm) baskets. Boxes with red lines represent spine loss for deep baskets, while blue is for shallow baskets. The corresponding letters denote significant differences between treatments. The boxplot indicates the spread of data in each treatment with the bottom and top-most reach of each line representing the maximum and minimum data of the treatment and the thick central line representing the median.

2.3.2 Effects of basket removal and depth on spine loss

Regardless of basket depth, removing baskets containing *Tripneustes gratilla* from the water column for five minutes resulted in significantly higher spine loss (0.341±006 g) than a short removal period of five seconds (0.089±0.001 g; $F_{(5,\,12)} = 31.7$, $p < 0.001$) or if not removed from the water at all (0.072±0.001 g; $F_{(5,\,12)} = 31.7$, $p < 0.001$). There was no significant difference in spine loss between short removals and no removals (Fig 2.4, $F_{(5,\,12)} = 31.7$, $p = 0.386$). While deep baskets removed from the water for long periods of time resulted in higher spine loss than shallow baskets, there was no significant difference ($F_{(5,\,12)} = 31.7$, $p = 0.100$). There was also no significant difference in spine loss from urchins between deep or shallow baskets when baskets were not removed from the water ($F_{(5,\,12)} = 31.7$, $p = 0.3528$). Shallow baskets resulted in higher spine loss than deep baskets when removed from the water for a short period of time ($F_{(5,\,12)} = 31.7$, $p = 0.007$).

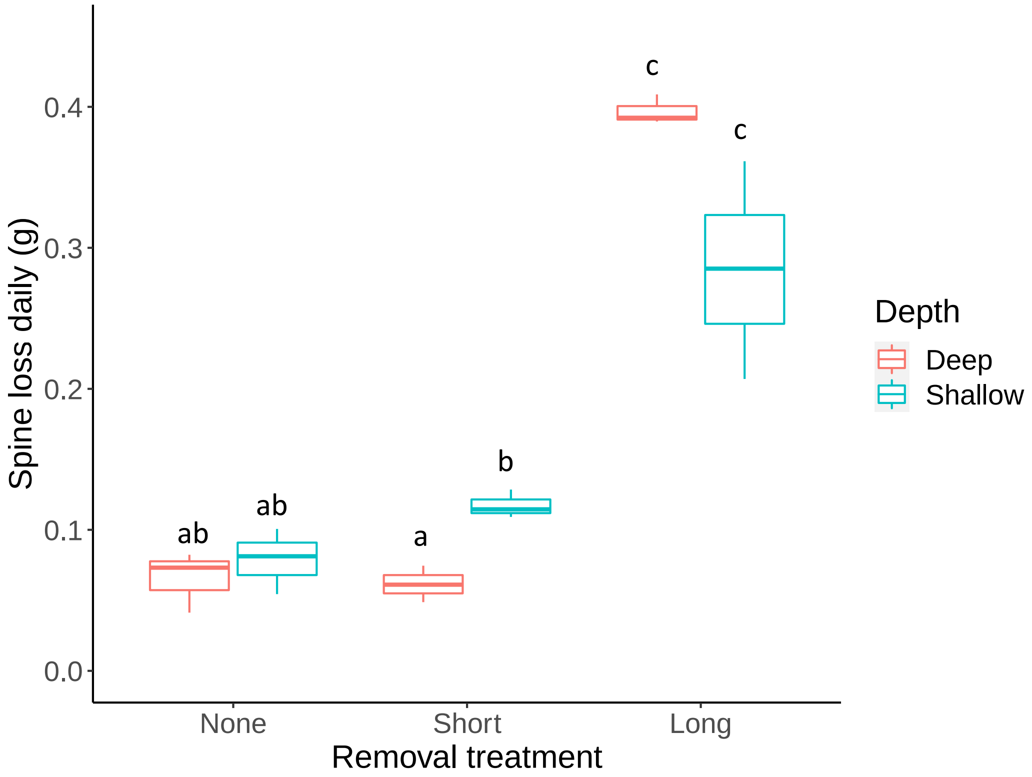
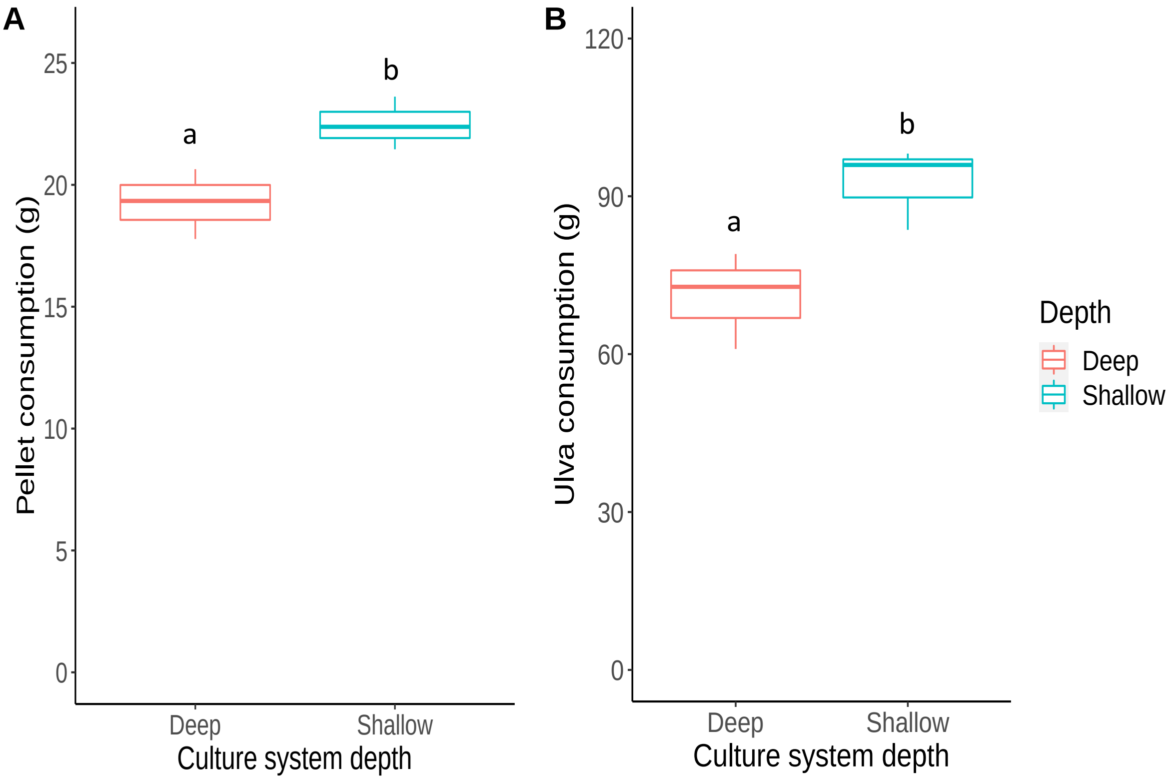


Fig. 2.4. Average daily spine loss (g) from *Tripneustes gratilla* maintained in deep (30cm) and shallow (15cm) baskets in response to three different removal treatments: none (not removed from the water), short (five second removal daily) and long (five-minute removal daily). Central line of boxes indicates median, box and whiskers represent interquartile ranges. Different lowercase letters above box plots represent significant differences between the mean spine loss of urchins housed in deep or shallow baskets within each removal treatment.

2.3.3 Effects of basket depth on consumption

Daily pellet consumption was significantly (*W* = 76.5, *p* = 0.007) higher in shallow baskets (22.48±1.176 g) than in deep baskets (19.26±2.058 g; Fig. 2.5.A). These values translate to a consumption rate of 1.23% and 1.05% dry pellets per wet urchin body mass per day, respectively.

The average daily consumption of fresh *Ulva* (Fig. 2.5.B) in the shallow baskets (92.7±61.15 g of fresh *Ulva* or 5.05% of BW.d^{-1}) was also significantly higher (*W* = 38, *p* = 0.026) than in deep baskets (70.94±84.10 g of fresh *Ulva* or 3.99% of BW.d^{-1}). This translates to a dry *Ulva* consumption to body weight ratio of 0.83% and 0.63% for shallow and deep baskets respectively.



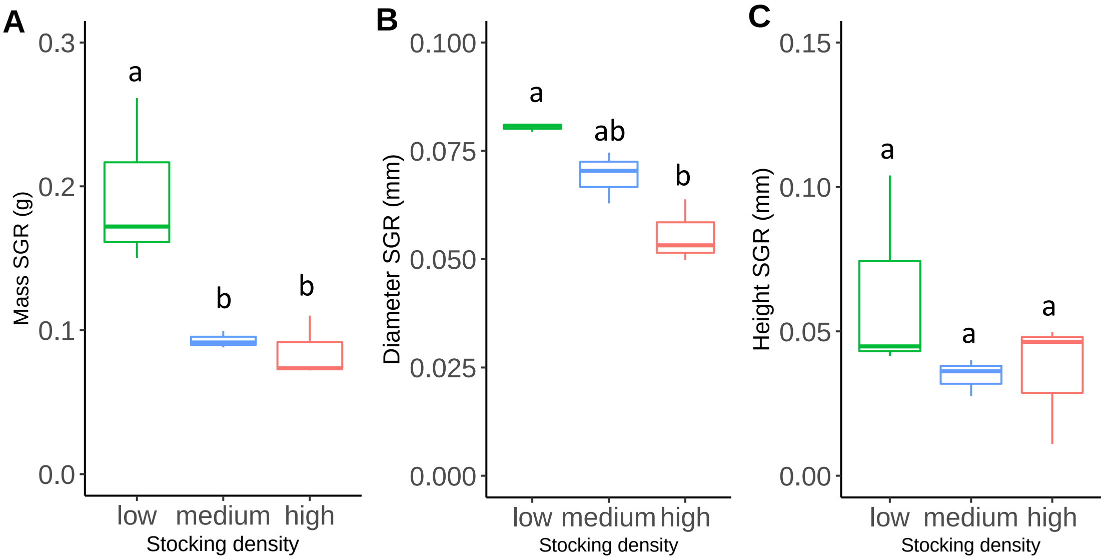
Figs. 2.5 A-B. Box plots comparing average daily consumption of dry pellets (A) and fresh *Ulva* lacinulata (B) by 22 *Tripneustes gratilla* housed in shallow (15 cm) and deep (30 cm) baskets. Different lowercase letters above box plots represent significant differences in consumption of pellets between treatments.

2.3.4 Effects of basket height on mortality

Over the two-month study period of the basket height analysis, there were eight mortalities across all treatment groups, which accounted for a 2.02% overall mortality rate. Six of these mortalities occurred in the shallow baskets, whereas only two occurred in the deep baskets. None of the possible explanatory variables (depth, tank, removal, feed and their interactions) had a significant influence on mortality (GLM; $Z_{(17)} < 1.346$; $p > 0.179$). As such, using Akaike information criterion (AIC) comparison of various models was irrelevant and there was no statistical evidence that mortalities were influenced by basket height or the other possible variables.

2.3.5 Effects of stocking density on the grow out phase

The average starting mass of an individual urchin was 109.66 g. After three months of being fed *Ulva* ad libitum, the mean masses at the low (4 kg.m^{-2}), medium (6 kg.m^{-2}) and high (8 kg.m^{-2}) stocking densities were 147.61 g, 133.07 g and 130.3 g, respectively. Stocking density had a significant effect on mass specific growth rate (SGR, $F_{2,4} = 9.434$; $p = 0.031$). The low stocking density had an average SGR of 0.194 g.d^{-1}, which was almost twice as high (Fig. 2.6.A) and significantly different from the medium and high stocking densities ($p = 0.044$ and $p = 0.034$, respectively). There was no significant difference in mass SGR between medium and high densities ($p = 0.869$). Stocking density also significantly influenced SGR of diameter (Fig. 2.6.; $F_{2,4} = 15.458$; $p = 0.013$). There was a clear significant difference between the low and high densities ($p = 0.011$), and weak evidence of difference between the medium and high densities as well as the low and medium densities ($p = 0.079$ and $p = 0.137$, respectively). While one basket treated with a low stocking density displayed a relatively high SGR for height, most of the baskets, regardless of stocking density, had a similar height SGR of approximately 0.05 mm.d^{-1} (Fig. 2.6.C). This experiment provided no indication that the tested stocking densities had any effect on the SGR of *Tripneustes gratilla* height ($F_{2,4} = 0.936$; $p = 0.464$).



Figs. 2.6 A-C. The influence of low (4 kg.m^{-2}), medium (6 kg.m^{-2}) and high (8 kg.m^{-2}) stocking densities on the specific growth rate (SGR) of *Tripneustes gratilla* mass (A), diameter (B) and height (C) of *T. gratilla* fed *Ulva lacinulata* over a period of three months. Different lowercase letters above box plots represent significant differences between the mean SGR of urchins held at different stocking densities.

At the beginning of this experiment, the low, medium, and high stocking density baskets were stocked with a total average of 1609.88 g, 2502.49 g and 3129.58 g of urchins, respectively. At the end of the experiment, the baskets had a total mass of 2129.72 g, 3034.44 g and 3696.99 g, respectively (Fig. 2.7.B). The percentage cover of the low, medium and high stocking density baskets increased from 13%, 19% and 24% to 15.25%, 22.77% and 29.87%, respectively. The greater the initial stocking density, the greater the total urchin yield at the end of the experiment ($F_{2,4} = 46.653$; $p = 0.002$), with significant differences between each treatment (Fig. 2.7.B, $p < 0.033$). However, the total increase of urchin mass in each basket did not vary significantly across treatments over the three-month period ($F_{2,4} = 0.07$; $p = 0.934$), where basket weight increased by approximately 0.5 kg regardless of stocking density (Fig. 2.7.A). The CV in mass of urchins maintained at a low stocking density decreased by an average of 1.98%, while the CV in the medium and high stocking densities increased by 0.10% and 0.66%, respectively, over the same time period (Fig. 2.7.C). There were, however, no significant effects from treatments ($F_{2,4} = 1.029$; $p = 0.436$).

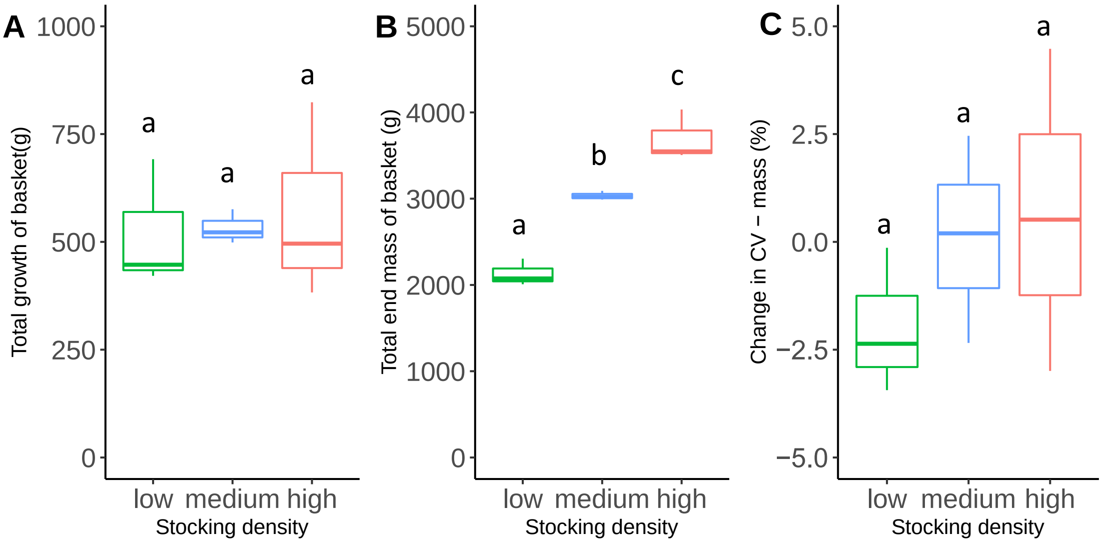


Fig. 2.7 A-C. The influence of stocking density on the difference of the total mass of all urchins in a basket (total basket mass) at the beginning and end of the grow-out experiment (A), the total basket mass at the end (B) of the experiment and the change in the coefficient of variation (CV) between urchins within each treatment from the beginning to the end of the experiment (C). Different lowercase letters above box plots represent significant differences between means of the stocking densities.

The mean gonadal somatic index (GSI) of urchins across all treatments at the end of the experimental period was 13.11%. The GSI data did not meet the assumptions required to apply any known statistical test correctly. A visual inspection demonstrated that GSI did not have a clear proportional relationship to stocking density. There is though, an apparent trend that GSI may become more variable with increasing stocking densities (Fig. 2.8.A). Similarly, the redness and yellowness did not meet the required assumptions for an ANOVA but there is indication these values may be reduced at the high stocking density (Figs. 2.8.C and 2.8.D). The lightness (L*) of the gonads did meet the assumptions and while there was some indication of an increase in lightness with an increase in density, this could not be supported statistically ($F_{2,4} = 0.286$; $p = 0.765$).

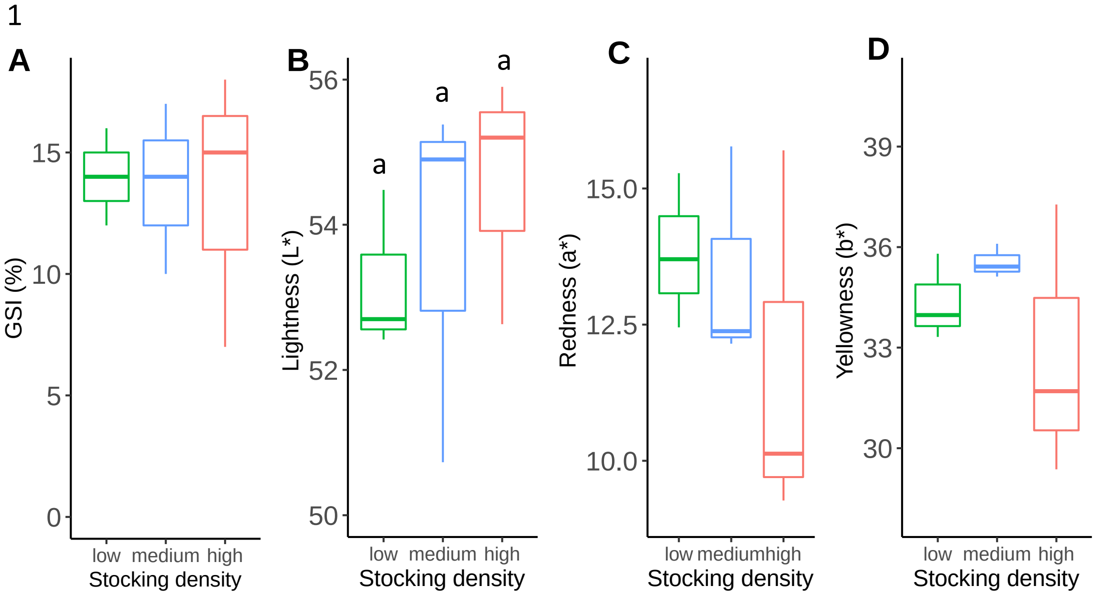


Fig. 2.8 (A-D). The effect of various stocking densities on gonadal somatic index (A), gonad colour lightness (B), gonad redness (C) and gonad yellowness (D) when fed *Ulva* over a three-month period. Matching letters denote a lack of significant differences in gonad lightness among treatments. Graphs lacking letters did not meet ANOVA assumptions and were excluded from statistical analysis.

2.3.6 Effect of stocking density on the gonad enhancement phase

At the beginning of the experiment, the average mass of an individual urchin was 169.69 g. By the end of the two-month period of being fed pellets every second weekday and *Ulva* over the weekend, the average individual urchin mass across all treatments was 185.41 g. There was evidence that stocking density had a significant effect on the specific growth rate (SGR) of mass ($F_{3, 9} = 5.048$; $p = 0.044$). The SGR in mass of individually housed urchins was significantly higher than that of urchins maintained at a high stocking density (Fig. 8.A, $p = 0.035$), whereas no significant difference in mass SGR was recorded between individually held urchins and those at the low or medium stocking densities (Fig. 8.A, $p = 0.110$ and $p = 0.066$ respectively). Furthermore, there was no significant difference between the treatments (low, medium and high stocking density) when multiple urchins were maintained in a single basket ($p > 0.647$). There was no clear statistical evidence that stocking density affected growth of urchin diameter (Fig. 8.B, $F_{3, 9} = 3.412$; $p = 0.093$) or the difference in coefficient of variance from the beginning to the end of the experiment (Fig. 8.C, $F_{3, 9} = 1.121$; $p = 0.412$).

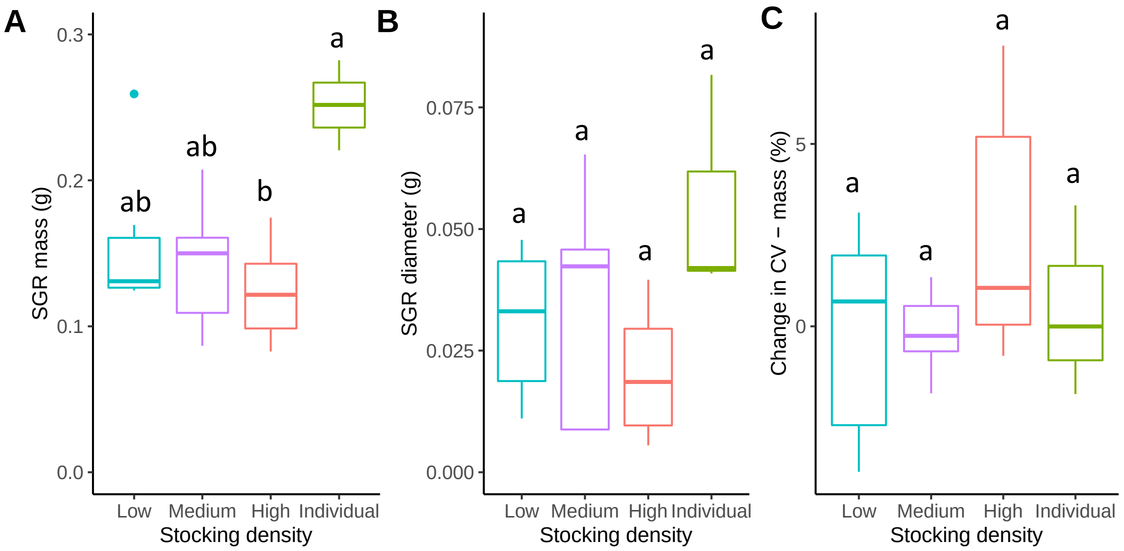


Fig. 2.9 A-C. The effect of stocking density on weight gain (A), size (diameter) gain (B) or change in the coefficient of variance (CV) of the mass (C) of *Tripneustes gratilla*. The contrasting letters in Fig. A indicate a significant difference between treatments. There were no significant differences in the data for Figs. B and C.

While the number of urchins in each basket between treatments differed greatly, the net increase in weight of each basket from the beginning to the end of the experiment was not significantly affected ($F_{3,9} = 1.765$; $p = 0.254$) (Fig. 9.A). There was high variation in GSI across all treatments. At the beginning of the experiment, the average GSI of the urchins was 9.15%, whereas by the end of the experimental period the average GSI across all treatments was 12.16% (Fig. 9.B). However, GSI of *T. gratilla* was not influenced by stocking density ($F_{3,9} = 0.594$; $p = 0.642$). The histological analysis revealed that most of the gonads examined from urchins were in a premature stage (stage 3), as described in Cyrus *et al.* (2015), regardless of stocking density (Fig. 9.C; $F_{3,9} = 1.875$; $p = 0.235$). Conversely, 6.7% of animals were in the recovery, partially spawned, or spent stages. There was no indication that stocking density influenced the colour (lightness (L*), redness (a*) or yellowness(b*)) of the gonads ($F_{3,9} > 0.781$; $p > 0.546$).

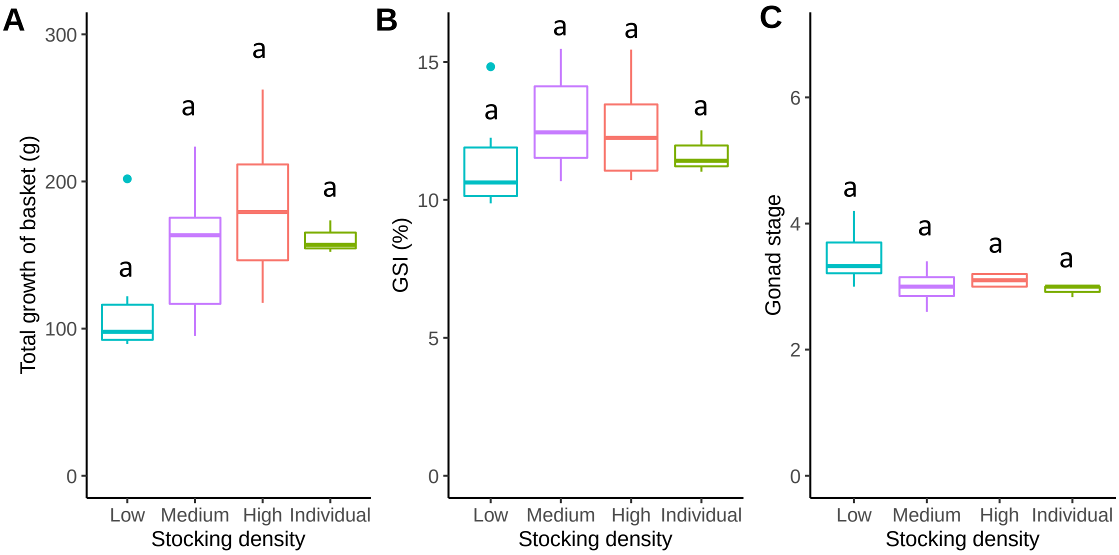


Fig. ***2.10*** **(A-C).** The influence of various densities of *Tripneustes gratilla* on their total basket mass increase (A), GSI (B), and average gonad stage per basket (C) over a period of two months while being fed pellets with 20% *Ulva lacinulata* inclusion. Different letters above bars represent significant differences.

2.3.7 The effect of stocking density on urchin behaviour (consumption, faecal production and spine loss)

The average daily consumption rate of *Ulva* by an individual urchin was 4.52% (wet weight) of their own body mass across all stocking densities (Fig. 2.11.A). Stocking density had no effect on the consumption of *Ulva* ($F_{2,9}$ = 0.833; p = 0.466). The percentage of faecal production relative to body weight (Fig. 2.11.B) also had no relationship with stocking density ($F_{2,9}$ = 0.639; p = 0.550). There was a significant correlation between faecal production and consumption (p = 0.017), but the relationship is not very strong (Pearson's correlation coefficient = 0.550). Daily individual spine loss, shown here as a percentage of urchin body mass (Fig. 2.11.C), significantly increased at higher stocking densities ($F_{2,9}$ = 9.551; p = 0.005). In a basket stocked at a high density, the individuals on average lost spines totalling 0.03% of their body weight daily, with a maximum of 0.04%. This was significantly higher than spine loss recorded in the low stocking density baskets (p = 0.005), which averaged at 0.013%. The medium-density baskets were not significantly different to the low- or high-density treatments (p < 0.124).

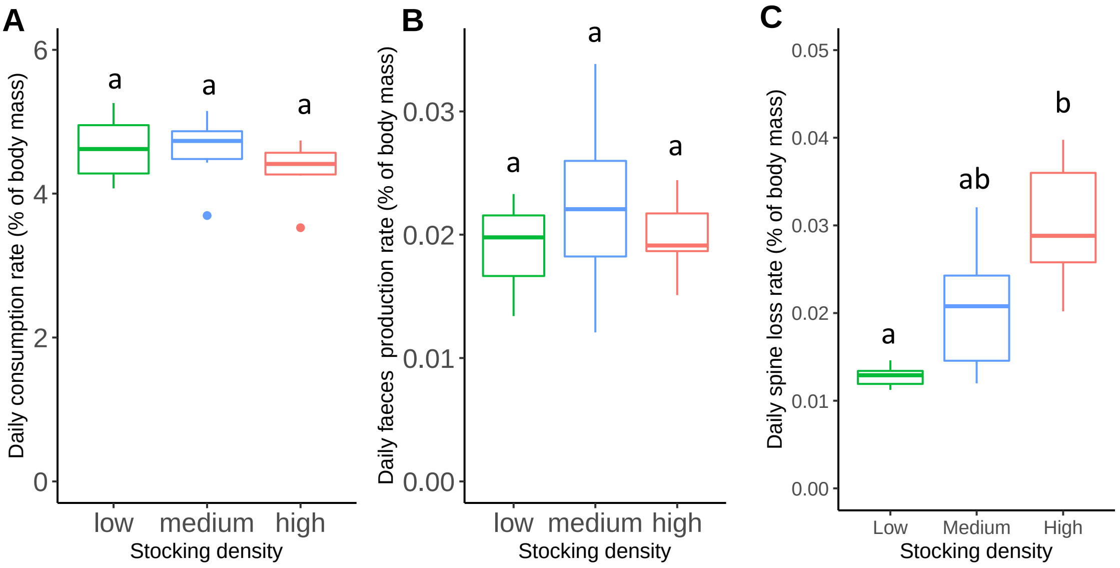


Fig. 2.11 (A-C). The influence of stocking density on various daily consumption, faecal production and spine loss. Different letters indicate significant differences between treatments.

2.3.8 Water quality monitoring

These analyses were conducted to test the assumption that varying production between treatments was primarily the result of behavioural interactions between urchins and not changes in water quality. Generalised linear models could not provide evidence that the number of urchins in a basket significantly influenced the pH or oxygen levels ($Z_{(26)} = 1.285$; $p > 0.200$). The dissolved oxygen remained almost constant throughout the experiment, at a mean of 78% and a minimum value of 75%. The inflowing water was also recorded and ranged from 7.87 to 7.61. The ammonium levels remained below detectable levels (< 0.005 mg/l) throughout the experiment.

2.4 Discussion

2.4.1 Effects of basket height and feed type on spine loss

This study found that deep baskets did not result in higher spine loss in *Tripneustes gratilla* due to collisions between individuals, contrary to Siikavuopio's (2009) theory. However, feeding kelp led to significantly higher spine loss compared to animals fed pellets or fresh *Ulva lacinulata*, regardless of basket height. The decomposition of kelp (*Ecklonia maxima*) in warm water caused mucus formation and affects water quality (Fleischman *et al.*, 2019; personal observation), but also leads to the breakage of spines when removing the kelp. This breakage occurs due to the firm, almost leather like, texture of kelp and when it is removed it runs along the test of the urchin and breaks its spines. Although the study couldn't quantify the impact of spine loss on growth reduction, previous research suggests spine loss negatively affects urchin reproductive and somatic growth (Ebert, 1968, 1967; Edwards and Ebert, 1991; Haag *et al.*, 2016). The observed spine loss in the kelp treatment could potentially reduce growth rates, emphasizing the importance of monitoring spine loss and avoiding degrading or firm feeds like kelp for echinoculture.

2.4.2 Effects of basket removal and height on spine loss

Spine loss was significantly higher in baskets removed from rearing tanks for longer periods, suggesting that prolonged removal results in more damage. As short removal periods (approximately 5 s) or no removals had no significant impact on spine loss, this suggests that maintenance practices, such as avoiding long removal periods, are crucial for reducing damage and maximizing urchin production. Preventing urchins from falling to the bottom of the basket when they are removed from tanks (which can be achieved by gently shaking the basket prior to its removal), may be more practical than adjusting basket heights.

2.4.3 Effects of basket height on consumption

Significantly lower consumption in deep baskets for both pellets and fresh *Ulva* provide a clear justification to culture *T. gratilla* in shallow baskets for optimal production (*ca.* 15 cm deep). This supports Devin's (2002) theory that urchin production is greater in shallow systems because the animals are largely sedentary and more likely to consume when feed is more accessible. Both pellets and *Ulva* sink to the bottom of the basket and the urchins prefer to attach to the upper portions of the basket. As such, in deeper baskets urchins may be less likely to travel the greater distance to the bottom of the basket to collect settled feed. For various urchin species, it has been observed that higher consumption results in faster growth rates (Cárcamo, 2015; McCarron *et al.*, 2009; Thompson, 1983). It can therefore be assumed lower consumption rates will result in lower production for *T. gratilla*. In the case of feeding *Ulva*, it may be possible to ameliorate this effect by keeping *Ulva* suspended by providing aeration directly beneath the urchin baskets.

2.4.4 Effects of stocking density on the grow out phase

There were no instances of mortality over the three-month experiment in any of the treatments. While higher stocking density reduced the individual growth of *Tripneustes,* it also allowed for a net increase in production (total mass per basket). Stocking densities of approximately 22.77% (medium) or greater significantly reduced the weight gain of individual urchins, while densities greater than 29.87% (high) significantly reduced the diameter SGR. The total mass of urchins by the end of the experiment, however, was significantly larger in the greater stocking densities. There was no clear evidence that stocking density influenced the quantity (GSI) or quality of gonads. These various metrics indicate the optimal stocking density will differ depending on the objective of the culture activity. If the objective is to have each individual urchin gain as much mass as possible, then a low density of approximately 15% cover is ideal. Conversely, if the objective is to gain as much total urchin mass as possible, a density of approximately 30% cover would likely be optimal. The objective will generally depend on the resource limitations of the aquaculture facility. For example, if juvenile supply and/or feed is limited or expensive, then low densities should be applied to maximise the potential of each urchin. If there are no limitations of feed, but only running cost and spatial limitations (number of baskets etc), then higher densities should be used. Each aquaculture facility will need to consider their specific limitations to determine their own optimal density. For simplicity, this study assumes the sole objective of the grow out phase is to increase the size of the test, thus providing a maximum test volume for the sequential gonad enhancement stage, as discussed in the introduction. The increase in height was not influenced by density but the diameter gain was significantly reduced when urchins were stocked above the medium stocking density. This medium density was initially stocked to 19% cover and ended as 22.77% cover. Therefore, this study suggests that if there are no resource limitations on the facility, a stocking density of approximately 20% cover should be used during the grow out phase of a *T. gratilla* production cycle.

2.4.5 Effect of stocking density on the gonad enhancement phase

There were two cases of mortality (0.98% mortality over two months), one in a basket with a medium stocking density and the other with a high density. These events occurred shortly after the experiment began and were therefore probably the result of handling stress. This suggests stocking density up to the high-density levels put forward by this study, that is, approximately 21% cover, and possibly higher, will not contribute to mortality. The individually housed urchins had significantly greater mass growth than those in the high-density treatment, but there was no significant difference between the high-, medium- and low-density treatments. As growth of the individually housed urchins will not be influenced by behavioural interactions, this implies that negative behavioural interactions induced by greater stocking density will only begin to limit urchin growth when density exceeds approximately 15.24% cover, while being fed pellets. It should be noted that while significantly different, the extent of this difference between stocking densities is very small. Furthermore, the mass SGR was the only metric (based on individual urchins) that showed a significant difference between stocking densities. The objective of the gonad enhancement period is to produce a high quantity and quality of urchin gonads, and there was no evidence of individual metrics related to gonads (GSI, colour or gonad stage) being influenced by stocking density. This implies that urchins could be stocked to the high density (21.06% cover) or possibly greater during the gonad enhancement phase.

2.4.6 The effect of stocking density on urchin behaviour (consumption, faecal production and spine loss)

There were no differences in the coefficient of variance of mass between stocking densities during both experiments. As such, there is no evidence that variation between individual urchin mass within experimental units was significantly affected by stocking density. There was also no evidence that differences in production between stocking densities were the result of changes in consumption. The clear relationship between spine loss and stocking density implies that reduced performance at higher stocking densities was likely due to negative physical interactions between urchins. There is substantial evidence that spine loss in urchins negatively impacts gonadal and somatic growth (as previously described). A similar observation was made in a stocking density analysis of *Strongylocentrotus droebachiensis* (Siikavuopio *et al.*, 2007).

2.4.7 Additional observations and improvements

The observed low SGRs in this study relative to previous studies can be attributed to the urchins being beyond their optimal growth phase (Dafni, 1992; Shpigel *et al.*, 2018) and the low pH recorded in the system which is discussed in depth in Chapter 4 (Mos *et al.*, 2015; Shpigel and Erez, 2020). The influence of behavioural interactions on stocking density is assumed consistent across different ages of urchins (personal observations), thus the findings in this study are still relevant for stocking density recommendations, especially when using a percentage cover metric. Water quality was recorded, finding no significant differences between treatments or blocks (tanks) implying the contrast in urchin production between stocking densities was the result of behavioural interactions and not a confounding factor. The gonad size observed in the gonad enhancement trial was lower compared to a previous study (Cyrus *et al.*, 2015), which used feed with an identical composition. This contrast between studies may be due to feed stability issues with this particular feed formulation, as experienced in another study (Shpigel *et al.*, 2018). Since all urchins in this study received the same pellets, the stability of the feed was not a confounding factor in this study. The observed metrics may not be absolute but still correlate, implying that the optimal stocking densities and management recommendations identified in this study are applicable in more ideal aquaculture conditions.

2.4.8 Conclusion

This study successfully achieved its objectives by determining the influence of basket depth and stocking density on the production of *T. gratilla*. It is found that while basket depth does not influence the spine loss or mortality it does affect consumption, which is reduced in deeper baskets. Subsequently, this suggest shallower baskets (*ca.* 15 cm) should be used to optimise the production of *T. gratilla* and possibly other urchin species. These experiments on basket height revealed additional insight that are valuable recommendations to *T. gratilla* aquaculture operators. The choice of feed significantly impacts spine loss, suggesting large and firm feeds such as kelp may not be optimal for *T. gratilla* production. Furthermore, it is emphasized the importance of handling baskets by demonstrating that removing them from the water for extended periods can damage the urchins and should be avoided.

When addressing the stocking density limitations due to behavioural interactions of *T. gratilla* this study determined that an optimal stocking density of approximately 20% cover is ideal for both the grow out (*Ulva*-fed) and gonad enhancement (pellet-fed) growth phases. While individual growth slightly decreased at this stocking density, it had no significant impact on mortality or gonad quality and led to greater net production of urchins. The results of this study offer practical guidance that can be of significant value to *T. gratilla* producers, ultimately playing a role in the advancement of more sustainable, ethical and economically viable practices for urchin aquaculture.

This study has demonstrated *T. gratilla* production will be enhanced in shallow baskets, stocked to at least 20% cover, and fed fresh *Ulva* or pellets. These variables had not yet been clarified in the literature but are fundamental to determine the financial, environmental, and functional feasibility of future urchin-*Ulva* IMTA systems. For this thesis, these are obligatory input variables required to predict the influence urchins will have on their water quality culture system, specifically the total ammonia nitrogen.

Chapter 3: Combining computer vision and standardised protocols for improved measurement of live sea urchins for research and industry

Abstract

For sea urchin aquaculture to be feasible on a commercial scale, an efficient and precise method of quantifying large amounts of urchins needs to be developed. This study demonstrates that the techniques generally applied are highly time-consuming, therefore completely impractical in a commercial context, and have high measurement error. Basic changes in these current techniques can considerably improve data quality. For example, urchin wet mass can vary up to 8.73% depending on time out of water, which is significantly reduced by allowing urchins to drip dry for at least 90 seconds prior to weighing. This study found the conventional vernier calliper method used to measure urchin dimensions to be both time-consuming and imprecise (mean coefficient of variation of 2.41% for *Tripneustes gratilla*). Therefore, this study automated the process through the development of a computer vision program. This software uses a series of HSV filters, edge detection algorithms and distortions to measure the test (not including spines) diameter of multiple urchins. This program measures with higher precision (mean coefficient of variation of 1.55%) and exponentially faster, relevant to the quantity of urchins, than the manual method. This open-source software is highly accessible, not requiring any specialised equipment and can be performed with a mobile phone camera. This computer vision application is combined with simple procedures investigated in this study, such as the minimum drip time, to create an accessible and straightforward standardised protocol to determine wet mass and diameter of sea urchins efficiently and precisely. For a larger-scale context, this software could easily be incorporated into various tools, such as a grading machine, to completely automate various farm processes. Thus, this study has potential to assist issues relating to urchin quantification in both a research and commercial context.

3.1 Introduction

To successfully produce and develop cultivation methods for large quantities of *Tripneustes gratilla* and any other urchin species, precise and efficient measurement methods are essential. Currently, methods most frequently used to measure live sea urchins involves weighing animals individually and then measuring the test diameter and height with callipers, as described and conducted in Chapter 2. These methods are not only imprecise, as shown by this chapter, but also highly time-consuming and impractical on a commercial farm scale, where conceivably millions of urchins will have to be measured monthly for grading and re-stocking purposes (Chapter 5). The latter constraints are further exacerbated for fast-growing urchin species, such as *T. gratilla*. Therefore, to ensure the feasibility of a commercial scale *T. gratilla* production facility, more precise and efficient methods of measurement need to be developed.

Precise measurements of urchin mass and outer test dimensions are also fundamental in a research context that extends beyond aquaculture. The significant ecological, social, and economic importance of these echinoderms has spurred extensive research with approximately 64 300 scientific publications referring to them in the past 20 years (Google Scholar, 2022). For data between studies to be comparable, and to increase the precision and accuracy of data within studies, data collection protocols must be standardised and optimised. Standardised measurement methods for fish have long been established (Ricker and Merriman, 1945; Schreck *et al.*, 1990), and more recently techniques have been developed for sea cucumbers (Watanabe *et al.*, 2012). For sea urchins specifically, standardised protocols have been developed for the collection, handling and analysis of urchin coelomocytes, that is, the immune effector cells of sea urchins (Smith *et al.*, 2019). There are however currently no clear standardised scientific methods widely accepted for measuring the dimensions or mass of live urchins.

Precise and reliable measurements are fundamental in any data analysis, whether they are intended for scientific publication or used to make management decisions on an aquaculture farm. There are various factors influencing wet mass measurements of sea urchins, which are not directly related to their true biomass. While, these factors cannot be completely removed, the total measurement error can be minimized by reducing and standardising their impact. Wet mass is defined as the mass of the whole organism and can be measured when the organism is alive. This definition does not include surface water. An urchin emersed from a body of water and immediately weighed will have considerably more surface water compared to an urchin left to drip-dry for a set period. While it is impractical and detrimental to the organism to have all its surface water removed, the more standardised the quantity of surface water on each urchin, the more precise the measurement will be. Certain papers mention specific units of time they allow urchins to drip-dry before being weighed (Ellers and Johnson, 2009; Russell, 1998; Santos *et al.*, 2020; Selden *et al.*, 2009), but these papers do not all use the same units of time and many other papers do not mention how long the urchins had been emersed before being weighed. Furthermore, the urchin mass measurements are likely influenced by the release of fluid when emersed. *Strongylocentrotus purpuratus* is known to emit an “emersion fluid”, which has been shown to be over a third of the urchin's volume and has a significant influence on its wet mass (Burnett *et al.*, 2002). Similar observations for *Tripneustes gratilla* and *Parechinus angulosus* have been noted (personal observation). This response appears to be correlated to the orientation of the urchin, where the rate of emersion fluid released is considerably greater when the urchin is upside down. The release of emersion fluid, and the rate thereof will influence the wet mass measurements of urchins and its influence should thus be standardised as far as is possible. Feed residing in the digestive tract may also skew the mass of an urchins, which is why some studies recommend starving animals for a few days prior to weighing (Cyrus, 2013; Sonnenholzner-Varas *et al.*, 2019). Contrary to this, it could be theorised that the density of most urchin feed is similar to that of water and, as the volume of an urchin is fixed, the fullness of the stomach will not affect mass. In support of this idea, a study comparing the wet weight of a group of *Paracentrotus lividus* fed *Ulva lactuca* daily with urchins starved for 36 days found no significant difference (Arafa *et al.*, 2006). An objective of this study is to provide methodology for accurate wet weight measurements of urchins, and recommendations for reducing the impact(s) of these factors. Therefore, this study investigates three factors that may add unnecessary measurement error: namely surface water, emersion fluid and feed.

Determining sea urchin test diameter is also important and requires methodological development, both for research and industry. Some studies have shown that certain treatments have no significant influence on mass, but do significantly affect urchin test diameter (Cyrus *et al.*, 2015). Conversely, Cárcamo (2015) showed the opposite, which emphasizes the benefits of measuring both size and mass of urchins. Urchin mass can be used to estimate diameter and, similarly diameter can be used to estimate mass, with species- and possibly condition-specific equations derived from regression models (Balisco, 2015; Kawamata, 1997; Stuart, 1981; Suskiewicz and Johnson, 2017). Creation of these models do however require large, accurate and specific data sets of the diameter and mass measurements and make the assumption of dependence between these values, which may result in failure to detect significant differences between treatments as shown in Cyrus *et al.* (2015) and Cárcamo (2015). Estimating mass from diameter, or vice versa, will further reduce accuracy of data due to some natural variation between the mass and diameter relationship in urchin populations. Hence, although there may be a correlation between mass and diameter, this relationship has not been examined across all urchin species, potentially leading to overlooked discoveries and diminished data integrity. As such, this study suggests both the mass and size be measured.

Sea urchin size is generally quantified by measuring diameter and sometimes height using vernier callipers (Christiansen and Siikavuopio, 2007; Cyrus *et al.*2015; Daggett *et al.*, 2006; Devin, 2002; Ebert, 1968; Edwards and Ebert, 1991; Haag *et al.*, 2016; Mos *et al.*, 2016: Pearce *et al.*, 2002; Siikavuopio, 2009; Siikavuopio *et al.*, 2007). This measurement technique may not be precise because urchins are not circular but pentagonal. This means the recorded value of the "diameter" of an urchin will vary depending on which part of the pentagon is measured by the callipers. Furthermore, measuring a sea urchin test with callipers requires the blades of the callipers to be against the test, which means they must pass through the layer of spines. Because urchins actively attempt to protect their test with their spines, this is not only difficult and time-consuming, but also frequently results in spine breakage and loss. Spine loss influences resource allocation as urchins regenerate broken spines, thus resulting in reduced somatic and/or reproductive growth (Ebert, 1968; Edwards and Ebert, 1991; Haag *et al.*, 2016). Furthermore, handling of urchins increases stress and reduces behavioural and innate immune defence responses, which can lead to increased susceptibility to disease (Bose *et al.*, 2019). As such, handling of urchins should be minimised. Other methods of determining urchin dimensions have been applied. Measuring urchin test surface area via three dimensional laser scanners has been reported to have high accuracy, but requires the animals to be sacrificed (Shpigel and Erez, 2020). Urchin tests have been measured via photographs (Mos *et al.*, 2016), where the user manually selects the measuring points on an image. While this may be accurate, it is labour intensive and thus not appropriate for use on a commercial scale.

As the echinoculture industry develops and larger quantities of urchins need to be routinely measured and quantified, more practical, rapid and efficient methods of measurement will need to be developed. This chapter will explore methods for large-scale sea urchin measurements, such as total basket mass and computer vision. Computer vision technology extracts useful information from images or videos. Machine vision uses computer vision to trigger an action, such as automating a task. Both have been applied to and greatly optimised for measurement of specific organisms of aquaculture or fisheries interest, including fish size, condition and behaviour studies in aquaculture (Saberioon *et al.*, 2017), and for determining volume and mass of oyster (Damar *et al.*, 2007), scallop (AiGuang *et al.*, 2006) and sea cucumber (Liu *et al.*, 2015). Sea cucumber machine vision is now even being applied to "sea cucumber catching robots" (Ge *et al.*, 2018). Currently, there are no computer vision applications involving sea urchins. However, it has been identified as a possible tool for the valuation of spine colour to predict gonad quality and quantity (Mos and Dworjanyn, 2019). Computer vision can involve costly optical sensors, specific imaging requirements and complex coding. It can also be highly accessible. This technology can be conducted via mobile phone cameras and make use of robust, simple, and open-source software, which does not require expertise in programming. The more practical and accessible the application, the more likely it will be used.

The aim of this study is to compare various standard measurement techniques and a newly developed computer vision approach, and establish guidelines on the most precise, efficient, and accessible methodology to measure live urchins. Through repeated measuring and measurement comparisons, this study provides expected standard deviations for the various measurement techniques. While scientific data collection rules that measurements should be taken by a single operator, it is not always feasible for a single person to conduct this task when large quantities of urchins need to be quantified in a short space of time. Thus, this study includes measurement error values for multiple operators. The standard deviation values provided here can be utilised by anyone to conduct power analyses to assist experimental design for live sea urchin studies. This could further improve sea urchin research methods in any field or context.

3.2 Methods

This study primarily used the sub–tropical/ tropical urchin *Tripneustes gratilla*, which has been identified as highly suited to aquaculture (Cyrus *et al.*, 2015, 2014; Juinio-Meñez and Hapitan, 1998). The *T. gratilla* were produced from larvae and reared at the Department of Forestry, Fisheries and the Environment (DFFE) Marine Research Aquarium (MRA) in Cape Town, South Africa. The temperate Cape urchin *Parechinus angulosus* was utilised to further verify the applicability of the computer vision program. These animals were collected from the seashore in front of the same research facility. The urchins were held in baskets (as described in Section 2.2.3) with dividers, which created 6 even compartments per basket. Each compartment held an individual urchin. Compartments were labelled to ensure the correct urchin was quantified each time. These baskets were placed in tanks with water parameters suitable to the specific urchin species (as described in Section 2.2.1). This study was approved by the DFFE Aquaculture Animal Ethics Committee (AAEC) without prejudice.

3.2.1 Factors influencing wet mass

Nine *Tripneustes gratilla* of various size classes (14.92-218.63 g) were removed from the water. After 5 seconds, a tared weighing boat was placed beneath the urchin and weight was recorded to the nearest 0.01 g. Urchins were then removed from the weight boat. The water in the weigh boat was removed using a paper towel and the weigh boat was re-tared. After 30 seconds of being removed from the water, the urchins were returned to the weigh boat and remeasured as before. This process was repeated at 60, 90, 120, 180, 300, 480 and 600 seconds for each urchin.

To determine if recent feeding influences the wet mass of urchins, a group of *T. gratilla* (n=36; 14-219 g) were not fed for a week. These urchins were then removed from the water for at least 90 seconds, which was found to be optimal in reducing the variance of wet weight, before placing on a clean, dry weigh boat and weighing to the nearest 0.01 g. Following this, urchins were supplied with a known mass of aquaculture-grown *Ulva lacinulata*, equivalent to 4% of their body mass. After 24 hours, when most urchins had consumed all the feed, the urchins were weighed as described above.

The precision of measuring the total mass of a group of urchins in a basket was compared to the average mass of the urchins quantified separately with 5 repetitions (described in detail in Section 3.2.2). Each basket, containing 6 individually housed urchins, was weighed once in the morning, midday, and afternoon (to account for possible diurnal change in mass) after being removed from the water for a minimum of 90 seconds. Once the baskets were emptied of urchins, they were re-weighed after being removed from the water for at least 90 seconds to determine basket weight.

3.2.2 Assessing precision of manual diameter, height, and mass measurements

The same group of 36 urchins (described above) were measured in terms of wet mass, height, and diameter five times (i.e. five measurement sets). Measurements were performed by three people/operators. Operator 1 conducted three of the measurement sets, whereas Operators 2 and 3 conducted one measurement set each. Operators 1 and 2 were experienced in measuring urchins but it was the first time that Operator 3 had worked with urchins.

Everyone received the following basic instructions on measuring the urchins:

- The blades of the vernier callipers must be placed against the test of the urchins and not the spines;
- The callipers must be placed as centrally as possible on the urchin and the value recorded to the nearest millimetre;
- The wet weight must be measured to the nearest 0.01 g; and
- The scale must be zeroed between urchins, and the urchins must be removed from the water for at least 90 seconds before being weighed.

The sets of measurements occurred hourly, and the time taken to conduct the measurements was recorded. Operators were randomized with respect to measurement set. The operators read their readings aloud to a scribe.

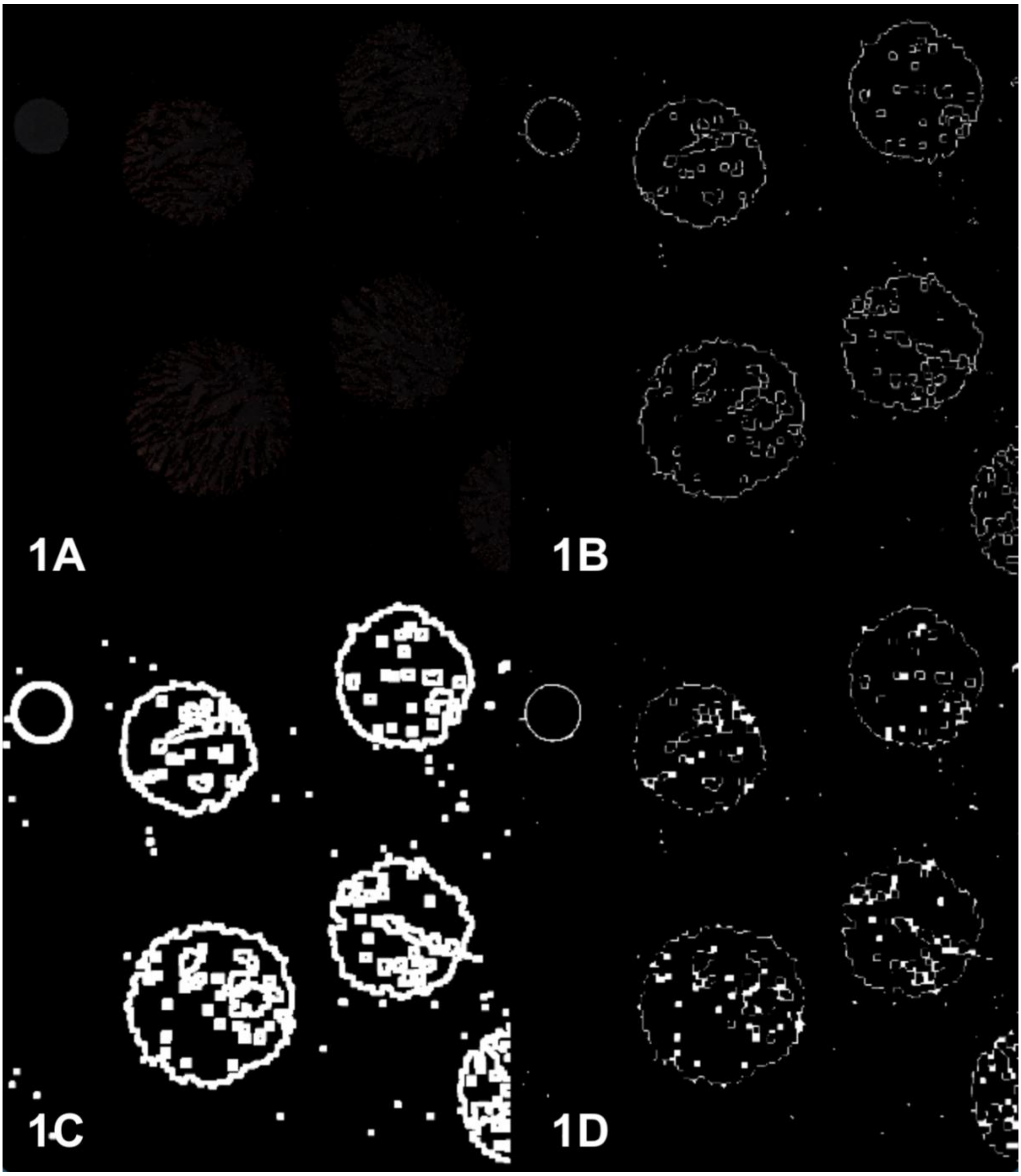
3.2.3 Computer vision

3.2.3.1 Hardware

To ensure accessibility of the computer vision program, no specialised equipment was used. All images were taken in a simple, homemade “photo box” using mobile phone cameras. The program does not necessarily need standardised lighting or distance between the camera lens and the specimen being measured. If the photo box designed for this study is copied it will increase the likelihood that the default light and distortion settings described below will appropriately capture contours, meaning the urchin's measurements will be accurate without needing to alter the settings of the program. The “photo box” was constructed from three Styrofoam boxes (70 × 35 × 18 cm, length × width × height) frequently used to transport seafood, which were stacked on top of each other. The bottom “floors” of the top two boxes were removed. To allow for the image to be taken, a small hole was cut in the centre of the lid of the top box. Urchins were placed aboral side up at the bottom of the lowest box. It was necessary to have approximately 1 cm gaps between urchins. A solid, black reference object with a known diameter was placed on the outermost left-hand side of the box, with no other objects placed further left of this object. Once the reference object and urchins were placed in the bottom of the container, the lid was firmly closed, before a mobile phone was placed on the lid with the camera directly above the central hole. The camera was set to a magnification of 1× without any photographic filters selected. There are no specific light sources required. More specific instructions on the camera setup can be found in Appendix G. To check for instrument bias, repeated images of the urchins were taken with two different mobile phones, a Samsung A52 and Huawei P30. To compare time efficiency between measurement methods (manual versus digital), the time taken to conduct this process was recorded, including the time taken to place the urchins and process images once appropriate parameters had been found.

3.2.3.2 Software

This program (de Vos and Batik, 2022) was written in Python3 (Van Rossum and Drake, 2009), primarily using the OpenCV library (Bradski, 2000). The complexity of applying computer vision to urchins involves distorting the image in a manner where only the test is measured and not the spines. A series of filters and constraints on the contour area were applied to achieve this. Initially, a hue, saturation and value (HSV) filter was shown to remove most spines (Fig. 3.1.A), predominantly via reduction of lightness. The image was then turned to greyscale and Canny contour detection algorithm (Canny, 1986) was applied to obtain an edge map (Fig. 3.1.B). Following this, a series of basic morphological operations (i.e. blur, erode and dilate) were applied to remove the last of the spines and smooth the image (Fig. 3.1.C). Once the distorted image represented the urchin test with sufficient precision (Fig. 3.1.D), the contours were determined using topological structural analysis by border following techniques (Suzuki and Be, 1985).



Figs. 3.1(A-D). A portion of an image of *Tripneustes gratilla* with the filters applied using default settings of the computer vision program to measure the diameter of the test not including the spines. Fig. 3.1.A is the initial HSV colour filter, mostly reducing the lightness. The following Fig. 3.1.B converted the previous image into greyscale and applied dilution filters and canny edge detection. Fig. 3.1.C applied erosion filters over Fig. 3.1.B. Fig. 3.1.D are the results of contours determined using topological structural analysis by border following techniques post colour and morphological images.

A minimum area bounding rectangle was then set around the complete contour of all objects identified in the image (Fig. 3.2). To quantify the size of each object, a pixel-per-metric ratio was determined through calibration of the reference object of known location and dimensions. The reference object was positioned as the left-most object of the image and its length (vertical diameter) was a required input. The program divided the number of pixels of the length by the known width of the reference object. This set a pixels-per-metric ratio, which was then applied to all lengths and widths of the bounding boxes around the objects in the image. As most mobile phones lack a truly flat lens, the shape of objects was distorted slightly where objects on the left or right extremes of the image have an apparent greater horizontal width than in reality. Objects on the bottom and top extremes have the opposite distortion. After extensive optimisation, it was found this distortion effect could be most effectively reduced by choosing the smallest value between the vertical or horizontal width of an object as the primary measurement value.

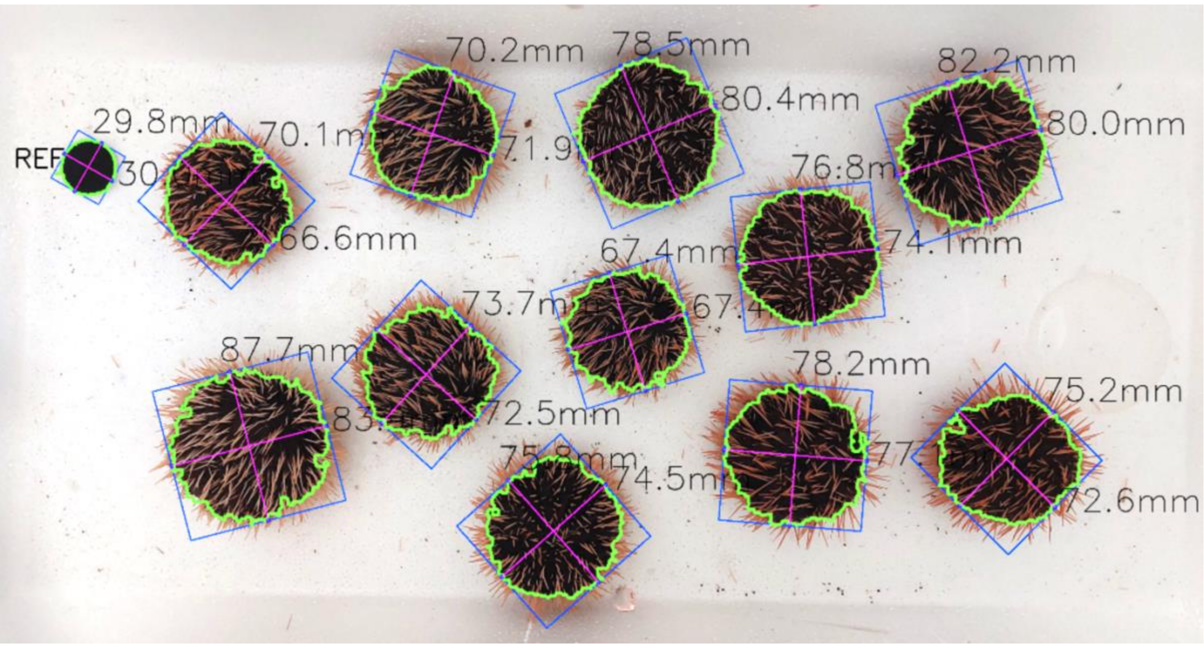


Fig. 3.2. Results of a full *Tripneustes gratilla* image once run through the program. Green lines demonstrate the edge detection, blue rectangles represent minimum area bounding and pink lines represent the widths. Note the reference object in the top left corner.

3.2.4 Assessing precision of computer vision-determined diameter

Tripneustes gratilla (N = 30) were each randomly labelled with the numbers 1–30 written on a small sheet of plastic that was attached to the urchin with an elastic band. These urchins ranged in size from 55.33 mm to 79.67 mm, as these were the sizes available at the time. The urchins were placed in a random order into the photo box and their image was taken as described above. The urchins were removed from the box and then immediately returned in different positions to the previous and their image was retaken. This was repeated three times, providing three measurements for each labelled urchin, thus determining the coefficient of variation. This exact experiment was repeated for 20 *P. angulosus,* which ranged in size from 15.17 mm to 58.17 mm. To provide an intraspecies comparison, the diameter of these 20 *P. angulosus* urchins was also measured using callipers, three times per individual, by the same operator.

3.2.5 Statistics

For all analyses was conducted in the statistical computing environment R (R Development Core Team, 2017). Excel was used to organize and present some data. The assumptions of independence and non-selectivity were met as discussed in the experimental designs. Significance was assigned to *p* values of < 0.05.

3.2.5.1 Factors influencing wet mass

To reduce the influence of urchin size, the mass of the first measurements (after 5 s of being removed from the water) was divided from all the measurements from each urchin and multiplied by 100 to transform data into a percentage of initial weight. No extreme outliers were observed as no data points exceeded the interquartile range by 1.5 times. Normality was found for each group by Shapiro–Wilk tests ($p < 0.026$). The assumption of data sphericity was met (Mauchly's test = 0.34). One-way repeated-measures analysis of variance (ANOVA) was applied to detect a significant effect of time on urchin wet mass, and a Bonferroni pairwise t-test was applied to detect significant differences among time intervals. A logarithmic function was fitted to this data to determine the possible presence of a near-constant mass (asymptote).

The paired mass data between the pre-fed and fed urchins were shown not to be normally distributed by a Shapiro–Wilk test ($p = 0.013$); thus, an exact Wilcoxon signed-rank test was applied.

To determine the similarity between weighing the wet mass of urchins individually and urchins together in a basket, a paired t-test was applied. The data were normally distributed ($p = 0.360$).

3.2.5.2 Comparing urchin measurement methods

There is no ‘gold standard’ urchin measurement method that can directly compare alternative methods. Thus, to allow for simple comparative analysis, this investigation applied a statistical approach similar to that of Watanabe *et al.* (2012), where CV was applied as the primary quantitative tool. The CV is determined by dividing the SD of the repeated measurements by their mean. Unlike SD, this provides a measure of dispersion of measurements, which is standardised, thus allowing fair comparison between multiple data sets and metrics (Hervé, 2010). A one-way ANOVA and Tukey post hoc test were applied to detect significant differences among treatments. Height measurements from callipers were excluded from statistical analysis as this parameter had considerably higher variability than the other methods and could not meet the assumptions of an ANOVA. Once these treatments were removed, all assumptions were met for normality (Shapiro–Wilk test, $p = 0.097$) and homogeneity of variances (Levene's test; $p = 0.135$). To test the accessibility and consistency of the computer vision application, measurements of the same images, but from two different phone cameras, were tested for significant differences via a paired t-test. A paired t-test was also applied to compare the diameter measurements between the callipers and computer vision.

3.3 Results

3.3.1 Factors influencing wet mass

There was a decrease in mass between the first measurement, taken at five seconds, and all the following measurements (Fig. 3.3). The greatest differences observed were between the five second and the 600 second weighing, with an average decrease in mass of 6.49% and the greatest difference of 8.73%. A one-way repeated measures ANOVA revealed that time out of the water had a significant effect on mass ($F_{(8,40)} = 97.327$, $p < 0.001$). There were no significant differences between the 90 and 120, 120 and 180, 300 and 480 and 480 and 600 second intervals (adjusted $p > 0.05$), with all other time intervals being significantly different (adjusted $p < 0.05$). The average coefficient of variation (CV) and standard deviation (SD) of mass within individuals from the 5 to 600 second intervals were 2.18% and 1.74 g, respectively. Within the 90 to 120 second interval, it was 0.01% and 0.29 g then between 90 and 600 seconds it was 0.01% and 0.65 g. A logarithmic function accurately fitted the relationship between time out of the water and average mass of all urchins divided by the initial weight at 5 seconds ($y = -0.013\ln(x) + 1.0153$, $R^2 = 0.97$; Fig. 3.3). There were no mortalities or any clear indications of stress after this trial. The mass of urchins after being fed was reduced on average by 0.28%, although the difference was not significant ($p = 0.093$).

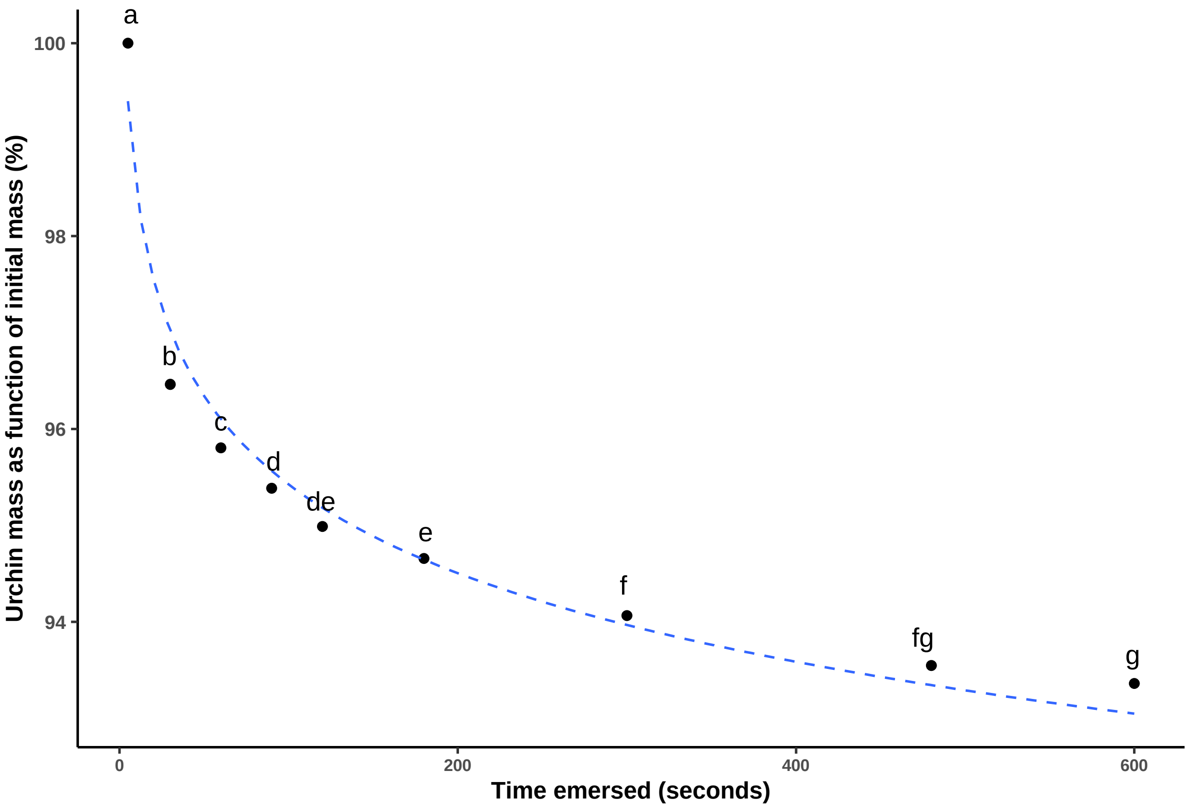


Fig. 3.3. A logarithmic function representing the percentage reduction of mass averaged between nine *Tripneustes gratilla* weighed at various times following removal from a body of water ($y = -0.013\ln(x) + 1.0153$, $R^2 = 0.97$). The percentage value was determined by dividing the mass of each urchin at individual time points by its initial mass at five seconds. Matching letters denote there being no significant differences in the average urchin mass between time emersed.

3.3.2 Measurement method comparison

The variability of height measured with callipers was considerably larger than the other methods (Fig. 3.4), where the CV was 3.98% when measured by the single operator and 6.46% when measured by multiple operators. There was a significant effect on CV when measuring test diameter with callipers, manually weighing urchins or determining diameter using computer vision tools ($F_{(4,187)} = 22.73$, $p < 0.001$). While the CV of diameter with callipers from a single operator (mean, 2.02%) was smaller than that of multiple operators (mean, 2.41%; Fig. 3.4), a Tukey post hoc test revealed no significant difference ($p = 0.23$). Similarly, there was no significant difference in CV for mass measurements between single and multiple operators ($p = 0.81$). Urchin mass determined by a single operator had the lowest mean CV (0.84%), which was significantly lower ($p = 0.002$) than the CV of the computer vision measurements (mean, 1.55%). Computer vision did not produce measurements significantly more variable than the measurements of urchin mass determined by weighing by multiple operators (mean 1.05%; $p = 0.073$). Similarly, the computer vision CV did not differ significantly from the measurements of the diameter using callipers by a single operator (mean 0.84%, $p = 0.066$). The entire process of manually measuring the diameter, height, and wet mass of 36 individual urchins took on average 42 minutes, which is approximately 70 seconds per urchin. The computer vision and total mass method of 25 urchins took on average nine minutes, including placing the urchin in the photo box, which translates to 21.6 seconds per urchin.

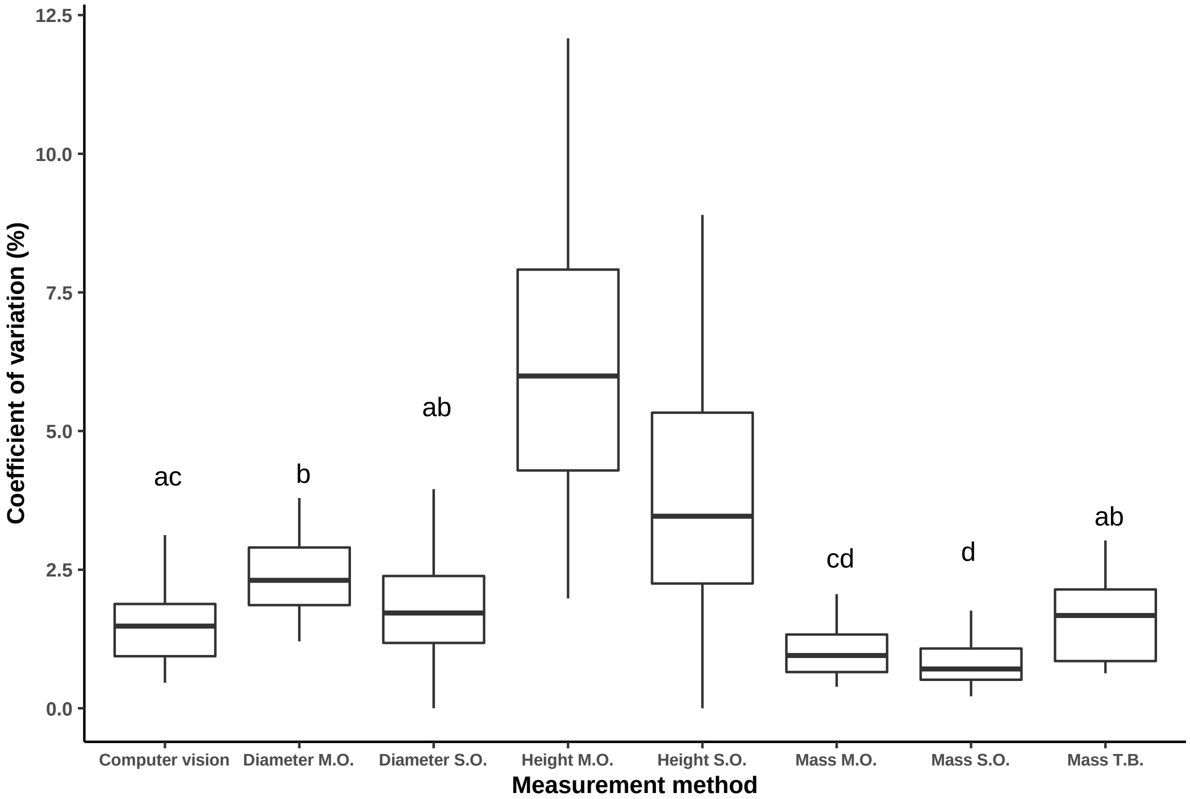


Fig. 3.4. Box plot comparing the distribution of coefficient of variation between various measurement methods for *Tripneustes gratilla*. The boxplot indicates the spread of data in each treatment with the bottom and top-most reach of each line representing the maximum and minimum data of the treatment and the thick central line representing the median. Treatments with matching letters did not have significantly different coefficients of variation from one another, there are no letters for height as it was not included in the statistical analysis. M.O. is an abbreviation for multiple operators, S.O. is a single operator and T.B. is for total basket mass, where all the urchins were measured together in their basket.

For *P. angulosus*, the CV for computer vision measurements, using the default imagine processing settings, was 2.21% and lower than that of all the manual measurements by a single operator with a CV of 2.65%, and there was no significant difference ($F_{(1,39)}$ =0.791, p = 0.379).

The images captured by the Samsung phone on average measured the diameter to be 0.18% greater than the Huawei, however, a paired t-test found no significant difference in measurements between phones ($t_{(29)}$ = 0.451, p = 0.655).

The average CV of total basket method for determining urchin mass (with empty basket mass deducted) was 1.67%, and when compared to the sum of individual urchin mass in the basket, the total basket mass (which was recorded by a single operator) was 3.26% larger and significantly different (p = 0.014).

3.4 Discussion

The findings of this study are used to recommend a protocol which uses both modern computer vision and basic instructions to significantly increase precision and decrease the time and effort required to quantify the average mass and test dimension of *T. gratilla* and other urchin species. This protocol should not only reduce handling stress and spine loss of urchins, thus preventing reduction of growth, but enhance the statistical power (likelihood of not detecting a significant difference, even though there is one) of urchin experiments. This could make the difference between meaningful results or vague deductions. Conducting an experiment on urchins where there are four replicates (baskets/groups of urchins), the minimal detectable difference between treatments is 2.5 mm in diameter and the significance level is 0.05 is applied as an example. If the calliper method with multiple operators is used, there will be a 56.83% chance of detecting a significant difference. With the computer vision method, for the same experiment, there would be an 88.4% chance of detecting a significant difference. This example demonstrates the importance of reducing measurement error, especially for time- and resource-intensive experiments. Standard deviations of various measurement methods are provided (Table H.1, Appendix H) and could be applied to conduct power analyses during experimental design.

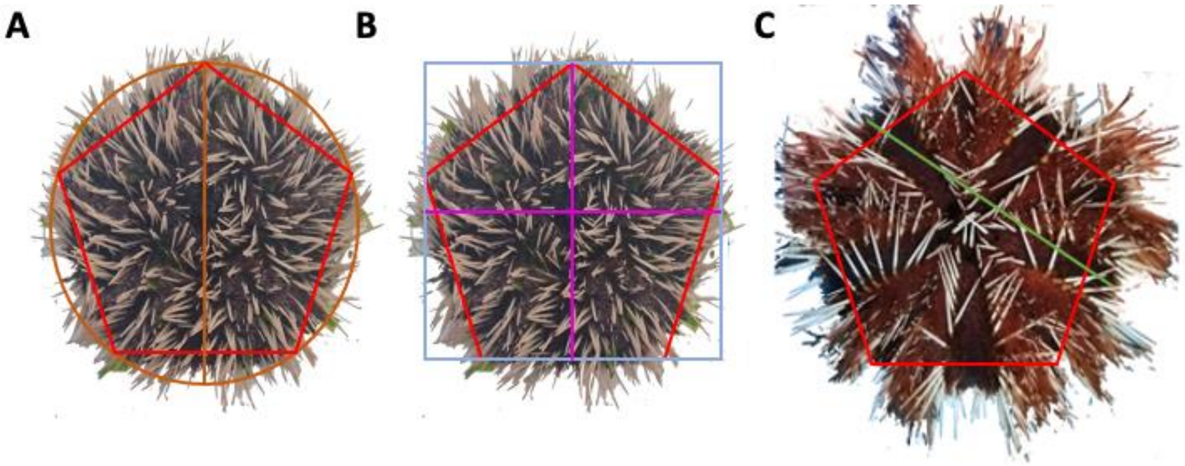
To achieve maximum precision of wet mass measurements, urchins should be removed from the water body/holding tank(s) and allowed to drip-dry for between 90 to 120 seconds before measuring weight (for reasons evident in results section 3.3.1 and in the discussion). However, this study suggests weighing urchins anytime between 90 seconds and 600 seconds after removal. This is because it may not be practical to measure urchins in this 30-second window and after 90 seconds most drip-loss had occurred. Water loss thereafter was negligible, with there on average only being a 0.01% change in mass between 90 seconds and 600 seconds. If a similar hypothetical experiment as previously described is conducted where the dependent variable is mass and the minimal detectable difference between treatments is 2.5 g, then waiting for at least 90 seconds gives a power value of 99.7%. Whereas measuring mass at any time from five seconds until 600 seconds after removal from the water would provide a statistical power of only 50.1%.

This study found no significant difference between mass before and after feeding, suggesting it is not necessary to purge animals (via starvation) to ensure mass is not influenced. Contrary to this, it may be beneficial to allow for a short purging period prior to handling and/or transport to reduce metabolism, as is done in fish aquaculture (Lines and Spence, 2012). This study observed a slight decrease in mass post-feeding and the reason for this is unclear. It may be related to possible differences in density between *Ulva* and coelomic fluid. Celomic fluid could be displaced externally as the urchin fills its digestive tract with feed (*Ulva*). While the biological and chemical properties of urchin celomatic fluid have been studied extensively, it appears no research has been conducted on its volume and how its volume changes. This could be worth further investigation.

Although not significant, variation between calliper measurements in diameter, height and for mass measurements was lower for a single operator than for multiple operators. While it is not always possible for a single person to quantify a vast number of urchins, it should be practised when possible and it is considered a basic protocol in scientific data collection to avoid operator bias. The total basket mass method for determining the combined weight of urchins in a basket did differ significantly from the combined mass of the individually weighed urchins. Therefore, this study suggests removing urchins from their basket but they can be weighed together as a group. It will be necessary to remove the urchins from their basket anyway to take an image for computer vision.

This study found the computer vision program, using default and generalist imagine processing settings, to be more precise and efficient when compared to measuring urchin diameter manually with callipers, as multiple urchins can be measured at once. Computer vision was at least 3 times faster than the manual method. Although measurement rates could vary for several reasons, the computer vision protocol developed in this study will very likely be exponentially more time-efficient with an increasing number of urchins. The manual method requires an operator and a scribe, while the provided computer vision protocol only requires a single person, halving labour requirements. The need for a scribe could however be mitigated by a have an electronic calliper and scale which can log the readings.

Urchin diameter measured by computer vision was shown to be significantly larger than diameter measurements using callipers. This is due to further limitations of the calliper method and not limitations of the computer vision program. As with all echinoderms, urchins are not round but rather pentagonal. Most spines occur on the vertices (corners) of the pentagon (Fig. 3.5.C). When measuring with callipers, the operator will generally avoid the spines and will measure from the edges (flats). As such, the calliper measurement will frequently not run through the centre on the urchins and therefore have a relatively lower value to the computer vision value. The pentagonal shape of urchins is one of the primary reasons the computer vision program used the minimum bounding area technique to create a rectangular shape on the extremities of an object and then determine the diameter of this rectangle (Fig. 3.5.B). In geometry, the diameter of a pentagon is the diameter of a circle drawn on the vertices of a pentagon (Pritchard, 2003). This means the "true diameter" of an urchin will be greater than the value given via calliper and computer vision measurements (Fig. 3.5.A), however the computer vision value should be closer to this true value. For the scope of this study and for the sake of measuring urchins, this is not too relevant and will not be an issue given the same measurement methods are compared. To clarify, direct measurements of outer test dimensions from computer vision and the calliper cannot be compared absolutely, however functions such as certain growth rates could be compared.



Figs. 3.5 (A-C). Various urchin 'diameters' are shown over the pentagonal shape of urchins (indicated by the overlayed red pentagon). Fig. A depicts the diameter as defined in geometry, where the length of the vertical brown line would be the diameter. Fig. B shows the diameter outputted from the computer vision program is the length and width (pink lines) of the minimum area building box (blue square). The green line in Fig. C demonstrates the measurement frequently made with callipers to avoid breaking spines. Note how this line does not cross the centre of the urchin. Different images of urchins were used to clearly demonstrate the shape (Figs. A and B) and then to show the distribution of spines (Fig. C).

The coefficient of variation of manual diameter measurements of *T. gratilla* was lower than *P. angulosus*. This is likely the result of *P. angulosus* being more challenging to measure accurately due to their harder, denser and longer spines, which increases the difficulty of getting the blades of the callipers against the test. This suggests that while the standard deviations provided in this study can be useful, some caution should be applied when conducting power analyses for different urchin species. The higher precision of the computer vision program with *T. gratilla* than *P. angulosus* was the result of colour variation between *P. angulosus* individuals (colours include black, orange, red, purple and white), which made it difficult to fit into generalist parameters of the colour filter. Regardless of this limitation, computer vision was still more precise than manual measurements of *P. angulosus* and now the default parameters, which were also analysed in this study, should work for urchins of most colours. There is still much scope for refinement and optimisation of the script for specific species. There was decreased precision due to mobile phones not having a truly flat lens. This could be improved by using more appropriate cameras, although this would reduce accessibility.

In conclusion, using total group mass to determine average individual mass after 90 seconds of drip-drying and then applying the computer vision program to determine diameter will provide precise measurements, with less disturbance to the urchins in considerably less time than previously used procedures. Manually measuring diameter with callipers is only a suitable method for quantifying small numbers of urchins. There is great potential to further develop the computer vision program. It can be further refined to improve the precision of measurements of different urchin species. This program could be extended to various machine vision applications, such as a grading machine. This protocol and new methods should assist anyone who needs to quantify a large group of urchins, regardless of their access to equipment and resources or whether the context is aquacultural, ecological or fisheries related.

Chapter 4: The effects of *Tripneustes gratilla* fed fresh *Ulva lacinulata* or formulated feed on water chemistry of an aquaculture system

Abstract

To achieve a functional and sustainable recirculating *Tripneustes gratilla* and *Ulva* integrated multi-trophic aquaculture (IMTA) system, a delicate balance of resource exchange is required. The specific design and management of this system required to attain this balance can be efficiently determined through farm-scale simulation modelling, which requires predicting the organisms' influence on water chemistry. While there is extensive literature on *Ulva*'s effects, little is known about *T. gratilla*. This chapter aims to understand the impact of *T. gratilla* and its feed on water quality parameters and provide training data for modelling its effluent chemistry.

A repeated measure factorial experiment was conducted where a series of tanks were treated to all combinations of presence or absence of *T. gratilla* and three feed treatments: namely, fresh *Ulva lacinulata*; formulated pellets with a 20% *Ulva* inclusion; and no feed. Six times over a period of 48 h, water samples were taken from the inflow and outflows of each tank. These water samples were analysed for total ammonia nitrogen (TAN), free ammonia nitrogen (FAN), nitrate, nitrite, phosphate, total suspended solids, alkalinity, dissolved carbon dioxide, temperature and pH.

The presence of urchins, feed treatment, time from feeding and their interactions had significant effects on TAN, nitrate, and pH ($F_{(1/2/5/10, 78)} > 3.303$, $p < 0.009$). In general, this suggests that for this emission model to be meaningful it must function on an hourly scale, be capable of factoring time from the previous feeding, and have feed types as input variables. The nitrogen concentrations in the *T. gratilla* effluent were generally low by aquaculture standards (average 0.001 mg/l TAN, 0.666 mg/l nitrate and 0.020 mg/l nitrite) and observed FAN levels would not reduce urchin production (maximum of 0.001 mg/l FAN). Phosphate concentrations were low (average 0. 005 mg/l) but substantially increased via leaching of pellets (average 0.05 mg/l), however remained beneath levels suggested to reduce urchin production. The total suspended solids in the effluent were minimal (average 0.142 g.l^{-1}). Feeding of urchins substantially reduced the pH of the tanks (minimum of 7.75). The levels of dissolved carbon dioxide were of greatest concern (maximum of 525 µATM), where the values observed in this trial may reduce urchin production. Additionally, management practices are recommended based on these findings, such as the necessity of regular sludge removal to avoid TAN accumulation.

This chapter provides both the baseline understanding and missing data set (as described above) required to model an *Ulva-T. gratilla* IMTA system. Additionally, these findings can be applied to any form of *T. gratilla* aquaculture to avoid water chemistry impacting the production of urchins and the environment receiving potentially toxic effluent.

4.1 Introduction

Interest is growing globally in the commercial culture of the tropical sea urchin, *Tripneustes gratilla*. Therefore, understanding and predicting how this echinoderm influences water chemistry in aquaculture systems is essential for numerous environmental, ethical and economic reasons. The most overt reason is to prevent urchins causing one or multiple water quality parameters to reach levels that negatively impact the organisms and reduce production. In aquaculture, this is traditionally avoided by replacement/dilution of the water within the system with water from the surrounding environment with excess wastewater generally returned to the surrounding environment as effluent. As this aquacultural effluent can be toxic and has caused severe environmental impacts (Martinez-Porchas and Martinez-Cordova, 2012), this provides another reason to understand the influence of *T. gratilla* on water chemistry prior to the establishment of a large-scale production facility. The quantity and quality of effluent can be mitigated through various means, such as *Ulva* biofiltration, as is a focus of this thesis. Regardless of the method of filtration, all have specific capacities and/or will only be effective within certain ranges of water quality parameters. Therefore, to correctly design and manage any wastewater remediation system requires some prediction of the effluent's water chemistry. For IMTA systems, accurate predictions are required to ensure conditions of the system achieve the aforementioned fine balance between the organisms, especially when efficient production is expected from multiple species, as is the case for the *T. gratilla-Ulva* system.

Water chemistry in aquaculture is difficult to predict as it is highly complex and influenced by numerous factors. The provided feed has a large impact on water quality and is often considered the sole source of nutrients (such as nitrogen and phosphate) in an animal aquaculture systems (Yogev *et al.*, 2017). Therefore, the emission of a specific element (such as nitrogen) in aquaculture effluent is frequently predicted simply by deducting the amount of the specific element supplied within the feed by the proportion expected to be retained by the fed organism. While these 'retention models' can be useful and/or more complex, they are frequently highly inaccurate as there are considerably more factors which will influence water chemistry (Wheaton *et al.*, 1994). A few of these factors, which basic retention models do not account for, include the microbial community (Bentzon-Tilia *et al.*, 2016; Fu *et al.*, 2015); particulate matter (Hargreaves, 1998; Klas *et al.*, 2006; Stewart *et al.*, 2006; Yearsley *et al.*, 2009); leaching from feed (Piedecausa *et al.*, 2010); and the size of the organism (Dy *et al.*, 2002; Kooijman, 2010). Therefore, the most robust method of understanding and predicting water chemistry of a system culturing *T. gratilla* is conducting an experiment which observes water chemistry parameters in various conditions similar to those expected in the proposed commercial systems. This approach is used in this chapter where the following water chemistry parameters are observed.

Dissolved inorganic nitrogen (DIN), including nitrate, nitrite, un-ionised ammonia and ammonium, is frequently the water quality parameter of greatest concern in aquaculture (Hargreaves, 1998). Dissolved inorganic nitrogen, specifically free or un-ionised ammonia nitrogen (FAN), is generally the first waste product of the fed organism to become toxic (Hargreaves, 1998). An equilibrium exists between FAN (NH_3) and the less toxic ammonium (NH_4^+), which is primarily driven by pH. At low pH, the balance shifts towards ammonium, while a high pH results in a higher ratio of FAN. FAN toxicity may limit urchin production (Hargreaves, 1998). While no studies have directly investigated the effects of nitrogen toxicity on *T. gratilla*, relevant data exists for other urchin species, such as *Strongylocentrotus droebachiensis* (Siikavuopio *et al.*, 2004b). These authors demonstrated that FAN at 0.680 mg/l resulted in a mortality rate of 76% over 42 days, while even a ‘low’ level treatment 0.016 mg/l resulted in a significant reduction in gonad size. This suggests that urchins have a low tolerance to FAN. Ammonia is converted into nitrate by two-step oxidation known as nitrification, which is primarily mediated by two bacterial genera, *Nitrosomonas* and *Nitrobacter*. This reaction is affected by multiple factors, including dissolved oxygen concentration, temperature, substrate concentration, pH, the number of nitrifying bacteria, and the availability of surfaces (Hargreaves, 1998). Nitrite may also limit *T. gratilla* production. Nitrite has no effect on mortality, even at a high level of 10 mg/l, but can reduce gonad size at levels as low as 0.5 mg/l in *S. droebachiensis* (Siikavuopio *et al.*, 2004a). Another study suggests nitrite levels should remain under 1-2 mg/l for *Paracentrotus lividus* (Basuyaux and Mathieu, 1999). The link between nitrite levels and reduced production is proposed as the result of a reduced feed conversion efficiency (Siikavuopio *et al.*, 2004a). Nitrogen, specifically total ammonia nitrogen (TAN, both NH_3 and NH_4^+), is usually the limiting nutrient for the production of *Ulva* (Solidoro *et al.*, 1997). Therefore, an understanding of the nitrogen flowing into an *Ulva* production system is required to design an efficient *T. gratilla*-*Ulva* IMTA system.

There is limited literature regarding nitrogen emissions of *T. gratilla*; while it provides some insight, it is insufficient to allow for accurate nitrogen emission predictions. Three ecological studies report *T. gratilla* (estimated average wet mass urchin of 80 g) collected from the wild would emit nitrogen at approximately 3.554 mg TAN.individual^{-1}.day^{-1} (Dy *et al.*, 2002; Dy and Yap, 2000; Koike *et al.*, 1987b). However, these values cannot be applied to accurately predict nitrogen emissions in the context of this thesis, due to this metric not accounting for the complexity of nitrogen emissions in aquaculture as previously described. These studies do still provide some relevant insight. Excretion rates of *T. gratilla* were greater during the day than at night (Dy and Yap, 2000). Smaller urchins had a higher metabolic rate than larger urchins, due to a negative correlation between ammonium emissions and mass (Dy *et al.*, 2002). The nitrogen emission of *T. gratilla* by mass was four-fold greater than that of the horned starfish (*Protoreaster nodosus*) and three-fold higher for brittle star (*Ophiorachna incrassata*; Dy and Yap, 2000). The average adult urchin nitrogen emission rate reported across various studies of various urchin species is approximately 0.234 mg TAN.individual^{-1}.day^{-1} (Arafa *et al.*, 2006; Asnicar *et al.*, 2021; Brockington and Peck, 2001; Hill and Lawrence, 2006; Wai and Williams, 2005). While the specific urchin size is not clearly reported in some of these studies, this average rate is over tenfold lower than the rates reported by the *T. gratilla* studies. This could be interpreted as *T. gratilla* having an exceedingly high nitrogen emission rate. However, this contrast may be the result of varying methodologies adopted in the different experiments. Unlike the studies of the other urchin species, Dy and Yap (2002) and Koike *et al.* (1987) specify the static water body that the urchins were held in was mixed prior to nitrogen analysis. This meant the solids settled on the bottom (sludge) would have been resuspended, which may contribute to the dissolved nitrogen concentration. Another, aquaculture-focused study investigated the effects of stocking and water flow rates on both adult and juvenile *T. gratilla* performance when fed *Sargassum* and denotes that the nitrogen emissions of *T. gratilla* is not substantial (Mos *et al.*, 2012). There were no detectable amounts of nitrite or ammonia produced (directly or indirectly) by *T. gratilla* even at stocking densities of up to 7.78 kg.m^{-2} and low flow rates of 0.3 tanks exchanges per hour (Mos *et al.*, 2012). The nitrate levels for *T. gratilla* were lower at higher stocking densities (approximately 0.02 mg/l) and lower flow rates (approximately 0.35 mg/l), even lower than the inflow (approximately 0.45 mg/l). This somewhat contradictory finding was attributed to nitrogen assimilation by the *Sargassum* and/or the microbiome within the tanks, further indicating the necessity of investigating these two factors. In overview, the composition, quantity and factors influencing nitrogen emissions from *T. gratilla* are unclear based on the current literature.

The reduced production in systems containing higher densities of *T. gratilla* and/or lower flow rates observed by Mos *et al.* (2012) were most clearly correlated to pH values. This reduction was the result of lower total alkalinity (A_T), calcite saturation (ΩCa) and increased partial pressure of dissolved carbon dioxide (pCO_2; Mos *et al.*, 2015; Shpigel and Erez, 2020). These three parameters were the only significant predictors in distance-based linear models for specific growth rate, linear growth rate and relative spine length. These factors are changed due to the uptake of carbonate ions for calcification, respiration and/or balancing of internal pH. Mos *et al.* (2015) suggests these changes in carbonate chemistry would lower *T. gratilla* production for a variety of reasons. These include lowering the availability of carbonates and therefore increasing energy expenditure of calcification (Doney *et al.*, 2009; Miles *et al.*, 2007; Stumpp *et al.*, 2012), decreasing the buffering potential of pH (Anthony *et al.*, 2011), hypercapnia (Pörtner, 2008; Widdicombe and Spicer, 2008), and disruption of regulation of internal pH balance (Miles *et al.*, 2007; Stumpp *et al.*, 2012). Other studies on sea urchins have shown similar results (Byrne *et al.*, 2013; Holtmann *et al.*, 2013; S. I. Siikavuopio *et al.*, 2007). Shpigel and Erez (2020) measured total alkalinity as a proxy for calcification of *T. gratilla*, and showed that adult *T. gratilla* assimilated calcium carbonate at a rate of 1.31 and 2.1 mg $CaCO_3$.individual^{-1}. hour^{-1} when fed an artificial feed and IMTA-produced *Ulva lactuca*, respectively.

Alkalinity also plays an important role in the pH-driven balance between the harmful FAN and less harmful ammonium, where low alkalinities result in lower buffering capacity (Boyd *et al.*, 2016). These relationships result in a myriad of complex and contradicting dynamics in the context of urchin farming, even more so when in co-culture with algae (such as *Ulva*). To prevent reduced production from high levels of FAN, steps could be taken to reduce pH. Reduced pH could though, result in a lower ability to calcify and various other negative impacts (discussed in chapter 2). Furthermore, when urchins are co-cultured with algae in poorly buffered water (<20 mg/l calcium carbonate), pH can exceed 9 in the late afternoon due to depletion of $C0_2$ via photosynthesis (Hariyadi *et al.*, 1994). This would result in nearly 40% of ammonia becoming un-ionized, as opposed to approximately 5% at a pH of 8 (Clegg and Whitfield, 1995). These dynamics need to be understood to successfully manage an urchin-*Ulva* IMTA system.

Phosphate is also relevant because low concentrations can limit *Ulva* growth (Waite and Mitchell, 1972). *Ulva* has, however, been found to assimilate phosphates at a relatively low-efficiency (Copertino *et al.*, 2009; Neori *et al.*, 1998, 1996). Dy and Yap (2000) reported adult *T. gratilla* emitted phosphate at approximately 4.32 mg.individual^{-1}.day^{-1}, which was significantly greater than the phosphate emissions of *P. nodosus* and *O. incrassata*. The tolerance of *T. gratilla* to phosphate is unknown, but a study on another warm water urchin species, *Lytechinus variegatus*, found that chronic exposure to even relatively low levels of phosphates (0.8 mg/l inorganic and 10 mg/l organic phosphate) could inhibit consumption, faecal production, nutrient absorption and growth (Böttger *et al.*, 2001). For context, total phosphate concentrations in monoculture intensive finfish recirculating aquaculture systems (RAS) frequently range between 25-45 mg/l (Ebeling *et al.*, 2005; Schneider *et al.*, 2006; van Bussel *et al.*, 2013).

Suspended solids are generally organic particles, derived primarily from feed or faeces that are light enough to be suspended in the water column due to turbulence, their small size and low density. total suspended solids (TSS) is an extensive water quality parameter because the solids can have varying compositions. As such, TSS has various important effects. It can have internal effects on aquaculture systems where high levels have been known to clog mechanical filtration systems and are correlated with high levels of nutrients (Schumann and Brinker, 2020). The most direct application of TSS is a simple, easy-to-determine and generalist water quality parameter that can be used as a benchmark to ensure that the effluent of an aquaculture facility has a limited influence on the surrounding environment. There are no records of TSS emissions values for land-based *T. gratilla* systems. However, a *T. gratilla* cage culture system (in the ocean) was found to have no effect on TSS levels in the surrounding environment (Malay *et al.*, 2000).

While the above literature provides some insights on how water quality is affected by *T. gratilla*, there are still substantial literature gaps which prevent comprehension of the dynamics and prediction of the composition of *T. gratilla* effluent from aquaculture systems. These gaps most fundamentally concern *T. gratilla* aquaculture when *Ulva* or formulated pellets (as described in Chapter 2) are fed. Both feeds are highly suitable diets for *T. gratilla* (Cyrus *et al.*, 2015, 2014; Shpigel *et al.*, 2018) but will likely have substantial influence on water chemistry. While extensive research has been conducted on the influence of formulated feed on water quality, its effect can vary depending on its stability, shape, surface area and composition of the formulated feed (Piedecausa *et al.*, 2010). This necessitates testing how this specific formulated feed developed for *T. gratilla* will influence water quality. Similarly with *Ulva*, there has been a significant amount of research into how it influences water quality when it is being cultured (in raceways etc.). However, it is not clear how it will influence water quality when it is being fed to *T. gratilla*. The influence *Ulva* has on water quality parameters is generally the opposite to the influence expected by urchins. Therefore, it is not clear what the net effect will be on water chemistry. While empirical evidence is required, this study hypothesizes that the *Ulva* will not be metabolically active, as the *T. gratilla* production is most likely optimal in low light conditions (as discussed Chapter 2). Therefore, it is unlikely to have a substantial influence on water quality by itself.

The aims of this study were therefore to:

- Create a training data set for regression models to predict the water chemistry parameters in *T. gratilla* effluent (when fed *Ulva* or pellets);
- Investigate the dynamics and primary drivers (explanatory variables) of water chemistry in *T. gratilla* effluent when fed *Ulva* or pellets; and
- Indicate which water chemistry parameters may reduce production of *T. gratilla.*

This will be a step towards accurate prediction and modelling of water chemistry in *T. gratilla* production systems, indicating conditional parameters (for example, feed rates and stocking density) that will not limit production. Furthermore, this will allow for estimations of water quality parameters of effluent. These can be used to indicate potential environmental impacts and provide insight into the application of mitigation strategies, with particular emphasis on a recirculating IMTA system using *Ulva*.

4.2 Methods

4.2.1 Overview

To assess the impact of different feeds (*Ulva lacinulata* and formulated feed, as described in Chapter 2) consumed by adult *T. gratilla* on aquaculture system water quality, a repeated measure factorial experimental design with 18 tanks was used. Half of the tanks housed *T. gratilla* while the other half remained unstocked. Within each group, three tanks with urchins received formulated feed, three received *U. lacinulata*, and three remained unfed as controls. The same setup was replicated for tanks without urchins. This design enabled the isolation of water chemistry changes caused solely by the feed and those influenced by urchin consumption. Additionally, an unfed treatment was included for both urchin and non-urchin tanks to serve as controls. The experiment ran for 48 hours over which six water samples were taken from all experimental units (tanks) at specific time intervals. These repeated measures from each tank allow analysis into how the time from feeding and time of day influences water chemistry. At each time interval, the water flowing into the tank and then the water exiting the tanks was sampled and analysed for TAN, nitrate, nitrite, phosphate, total suspended solids (TSS), calcium carbonate, dissolved carbon dioxide and pH. Specific details of this experiment are provided below.

4.2.2 Experimental set-up

This trial was conducted at the Department of Forestry, Fisheries and Environment's (DFFE) Marine Research Aquarium in Sea Point, Cape Town, South Africa from 18/02/2022 until 06/03/2022. Raw seawater was pumped from the seashore through a sand filter into a 5000 l header tank. It was passed through an ultraviolet filter into another 5000 l header tank where the water was heated to 27°C with a heat pump. From this tank it was pumped into each of the 18 urchin experimental tanks. Each 40l (0.04 m^{-3}) plastic experimental tank (30 x 42 x 32 cm, LxWxD) contained a 4 mm HDPE mesh basket (25 x 40 x 20 cm, LxWxD), which had an internal surface area of 0.36 m^{-2}. The water passed through each tank into a drain, making it a flow-through system. Flow rates for each tank was adjusted to 1.1±0.01 (standard deviation) tank water exchanges per hour. Constant aeration was supplied to each tank using a 4 mm HDPE pipe without an airstone. These tanks were indoors and did not receive any direct sun light but only mild ambient lighting on a 12 h light/12 h dark cycle. This was done to increase applicability of this study, where it is likely *T. gratilla* will be farmed in shaded or indoor conditions due to their sensitivity to light (Kehas *et al.*, 2005; Lewis, 1967; Muthiga, 2005; Park and Cruz, 1994; Ziegenhorn, 2016) and to prevent the growth of epiphytes in the culture system. The light levels were found to not exceed 3.33 $\mu mol\ m^{-2}s^{-1}$ (Volt Craft MS-1300 photometer, Conrad). To attempt to inoculate a microbial community likely associated with *T. gratilla* and the various feed treatments, all tanks were provided with four adult urchins and their allocated feeds (provided every 2nd day) for a week prior to sampling. These urchins were removed prior to the experiment.

Nine out of the 18 tanks were stocked with 16 *T. gratilla* urchins (ranging from 60 to 80 mm in diameter). The wet mass, height and diameter of each urchin were measured using a scale and callipers, respectively. The stocking density by available surface area was 6.36± 0.05 $kg.m^{-2}$ and by volume 57.34 $kg.m^{-3}$, reflecting a maximum that would be expected in a commercial system (Chapter 5). Twelve hours prior to beginning water sampling, the urchins were removed from the nine tanks and all 18 tanks were siphoned to remove all the sludge. The following morning, six tanks, three with urchins and three without urchins, were provided with 137.6 g of *Ulva*. This quantity equates to 6% of the total mass of the urchins, which a pilot study found to be the maximum amount of *Ulva T. gratilla* can consume in a 24-hour period. Another six tanks, three with urchins and three without urchins, were provided with 34.4 g of formulated feed (pellets) supplemented with 20% dried *U. lacinulata*, as described by Cyrus (2015a). This amount of formulated feed was determined as the maximum amount *T. gratilla* can consume in 24 h (1.5% of total biomass) based on preliminary trials. The urchins were only fed once in this trial. The remaining six tanks, with three of these tanks containing urchins and another three not containing urchins, were not provided with feed and therefore making up the unfed treatment. Tanks housing animals that were provided with feed were fed one hour before sunrise (5:30 am). The first sampling began an hour later (t+1). This process was repeated multiple times after the feeding: at six hours (t+6); 12 hours (t+12); 16 hours (t+16), that is, after sunset; 24 hours (t+24), that is, shortly before sunrise; and 48 (t+48) hours.

4.2.3 Water quality analyses

The pH and temperature were measured at each sampling interval (as described above) at both the inflow and outflow of each of the 18 experimental tanks, both the inflow and outflow were at the surface of the tanks at opposite sites. This was done using a probe (HOBO MX2501), which was calibrated at each sampling interval. A volume of 200 ml of water was taken from the inflow and outflow of each tank at each of the sampling time-points and filtered through a 0.7 µm glass microfiber filter. These pre-weighed filters were then dried to constant weight (60 °C) and re-weighed to determine the total suspended solids. Unfortunately, a sampling error occurred with the weighing of the filters and only a single-time sample was captured. The filtered water was decanted into three 50 ml and one 15 ml centrifuge tubes, which were frozen and then later used for further analysis. Salinity was measured using a refractometer and was found to consistently be 35ppm regardless of treatment or time.

Nitrate, nitrite and phosphate concentrations were determined with a SEAL AA500 AutoAnalyzer, following auto-analysis protocols (Grasshoff *et al.*, 2009). Detection limits of 0.1 µM for nitrate+nitrite, 0.05 µM for phosphate, and 0.01 µM for nitrite were applied. Nitrate concentrations were calculated by subtraction. Standards of various concentrations were run after every 10 samples to ensure instrument accuracy. Certified reference materials (KANSO) were included at the beginning and end of all analyses to ensure the accuracy of the measurements.

Ammonium concentrations were determined via the fluorometric method (Holmes *et al.*, 1999). Two separate 40 ml centrifuges tubes of each sample were analysed. The detection limit was 0.01 µM. The matrix effect that resulted from the calibration of seawater samples to Milli-Q standards was calculated according to the standard addition method (Saxberg and Kowalski, 2002). Equations were based on fluorometrics (Taylor *et al.*, 2007) and were always < 10%. All sample measurements were corrected for the matrix effect. To calculate FAN from ammonium concentration, a dissociation coefficient (pK_a) was determined, which factored in the temperature, pH and salinity at the time of sampling as outlined in Equation (4.1) (Bell *et al.*, 2007).

$$pK_a = 10.0423 - (0.0315536 \times T) + (0.003071 \times S) \tag{4.1}$$

Where:
T = Temperature (◦C)
S = Salinity (ppm)

Once pK_a was determined, the ammonium to ammonia concentration (mg/L) ratio was determined via Equation (4.2).

$$1 + 10^{(pKa\ -\ pH)} \tag{4.2}$$

Alkalinity was determined by using a filtered 15 ml aliquot from each sampling point, which was fixed with mercury chloride at two-time intervals (24 and 48 hours). The fixed samples were quantified for calcium carbonate concentration using a colourimeter (HI755 Hanna Instruments) using the reagent HI-755–26 and instructions provided.

The dissolved carbon dioxide was calculated via the Seacarb package (Gattuso *et al.*, 2021) in R, using their relationship with the observed concentrations of alkalinity, pH, temperature, phosphate and silicate using the constants defined by Lueker *et al.*, (2000).

4.2.4 Statistical analysis

Three-way repeated-measure analysis of variances (ANOVAs) were used to detect significant main and interacting effects of the within independent variables that influenced the water quality parameters (TAN, NO_2, NO_3, $P0_4$, TSS, pH, alkalinity), while controlling for the effects of time, since water samples were acquired at various points from each tank (experimental unit). The independent variables were the feed treatments (pellets, *Ulva* or not fed) and the presence or absence of urchins in the system. To allow for easy interpretation and comparison between treatments, two-way repeated measure ANOVAs were also constructed with the only difference being that two sets of independent variable categories were grouped into all the various combinations. These ANOVAs included an error term of the tank and time the sample was taken. If these ANOVA were significant highly conservative Student–Newman–Keuls (SNK) tests were applied to determine the presence of significant differences between treatments. Each dependent variable was checked for normality and sphericity using Shapiro-Wilks, box plot and Mauchly's tests/methods. There were instances when assumptions were only met with the appropriate transformation. Some outliers were present in the nitrite and nitrate data, but they did not influence any binary conclusions from the statistical analyses. This was checked by comparing models with and without outliers. The TAN, phosphate and alkalinity data had outliers ($n < 3$) that had to be removed as they were severe, and the samples were observed to have been taken during a spawning event and/or sampling errors (water samples contaminated with oil from pump used to filter water samples). Furthermore, the phosphate analysis had to be separated into pellets and without pellets as the contrasting magnitude of variance between the two sets did not allow for assumptions to be met. A mistake in the collection technique resulted in only a single set of TSS values (at t6), meaning this was not a repeated measure experimental design. As such, only a two-way ANOVA was applied.

4.3 Results

Ulva and pellets were all consumed within 24 hours and the first faeces from both feeds were first observed after eight hours. The temperature remained relatively constant throughout the sampling period with an average of 25.77±0.6 (standard deviation) °C. There was a single mortality eight hours after the urchins were fed pellets and there was some evidence of spawning prior to this mortality. Data from this tank were removed from statistical and visual analyses.

4.3.1 Total Ammonia Nitrogen (TAN)

The TAN concentration of inflowing water was on average 0.003±0.001 mg/l. The feed treatment (*Ulva*, pellets or not fed), the presence of urchins and the time when the water sample was taken relative to the previous feeding all had significant main and interacting effects on the TAN concentration ($F_{(1/2/5/10,\ 78)} > 3.715$, $p < 0.001$). The pairwise comparison SNK test provided no evidence that the tank itself influenced TAN concentration because there was no significant difference between TAN concentration of the outflowing water of the control treatment (tanks without urchins, pellets or *Ulva*) and the inflowing water ($p = 0.988$, Fig. 4.1). Furthermore, there was no significant difference between the TAN concentration of the inflow and outflow for tanks without urchins subjected to only pellets or *Ulva* ($p > 0.413$, Fig. 4.1). TAN concentrations were only influenced significantly when urchins were added to the tank (Fig. 4.1).

Effluent from tanks containing urchins that were unfed for the duration of the experiment had a significantly higher concentration of TAN than the inflowing water ($p = 0.001$, Fig. 4.1). There was no significant difference in TAN concentration between urchins fed *Ulva* or pellets ($p = 0.894$, Fig. 4.1). On the other hand, the TAN concentration of these treatments was significantly higher than unfed urchins, the inflow, and all other treatments ($p < 0.001$, Fig. 4.1). The fluctuations in TAN in the pellet treatments were severe (Fig. 4.2). The maximum recorded TAN value (0.031 mg/l) was over 10 times greater than the average TAN of the inflow, and occurred an hour after pellets were fed to urchins. The minimum observed value was 0.001 mg/l recorded 12 hours after pellets were provided to a tank without urchins (Fig. 4.2).

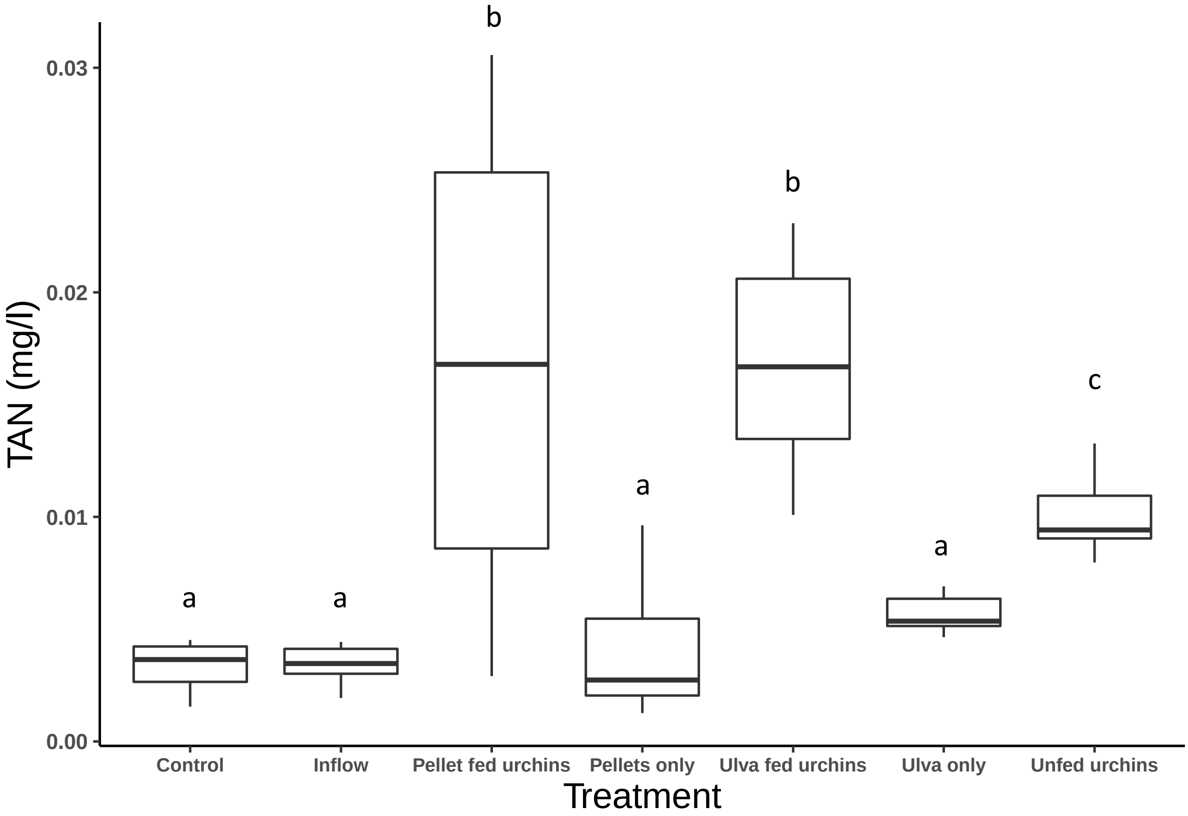


Fig. 4.1 Total Ammonia Nitrogen (TAN) concentration was compared in the inflow and outflow of tanks treated to various combination of *Tripneustes gratilla* presence/absence and feed types. All samples taken over the 48-hour experimental period were pooled within treatments to provide a broad perspective of their influence on TAN concentration. The letters correspond with the significance deduced from a Student–Newman–Keuls (SNK) test, where matching letters indicate that there were no significant differences between treatments.

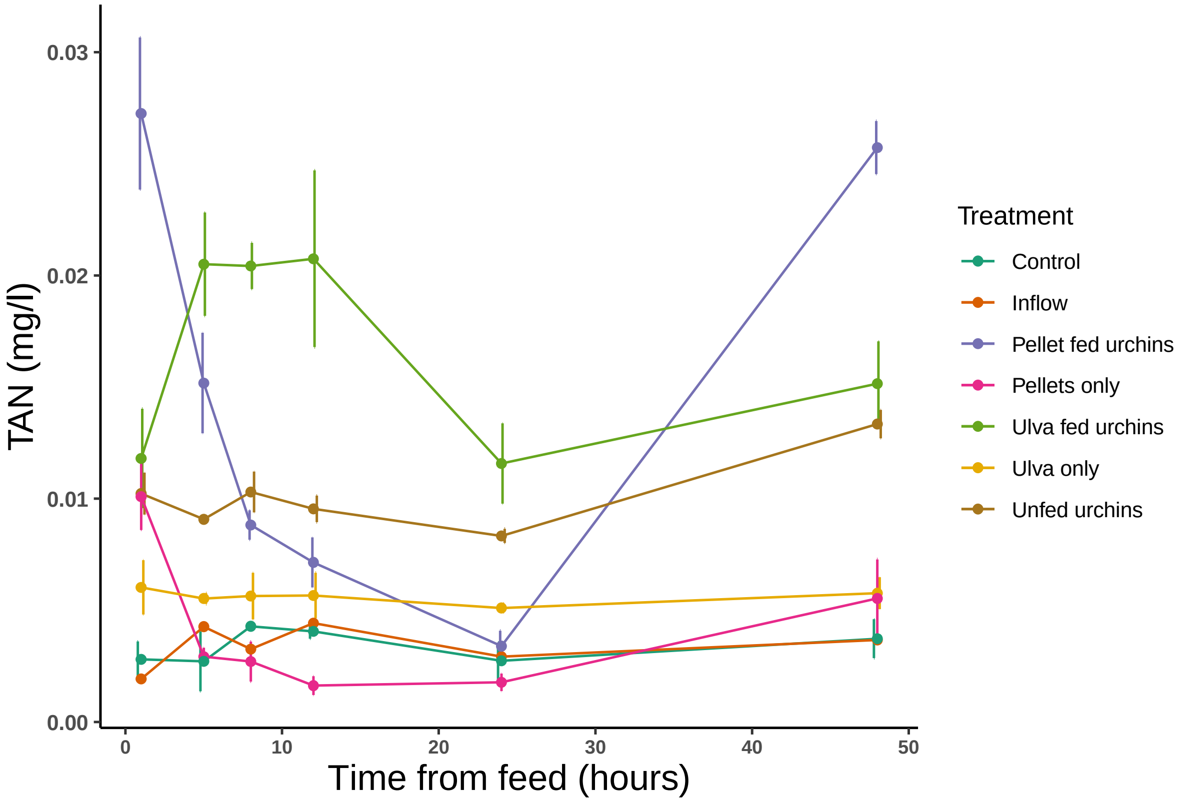


Fig. *4.2*. This shows the same total ammonia nitrogen (TAN) concentrations of inflow and outflow of the various treatments as Fig. 4.1 but demonstrated as a function of time. The data points represent the mean value of the treatments at the specific time interval and the vertical lines indicate the standard deviation observed between replicates. The vertical bars represent the standard deviation. The shaded blocks represent night time. The control is the three tanks that did not contain urchins or feed.

The relationships of FAN concentrations with time, presence of urchins and feed treatment largely mimicked those of the TAN data. The primary contrast was the maximum recorded FAN level (0.001 mg/l), which was observed 48 hours after pellets were fed to urchins (Fig. 4.3), instead of directly after pellets were fed. The initial differences between FAN concentrations of the *Ulva* and pellets fed were less than the differences observed in the TAN concentrations.

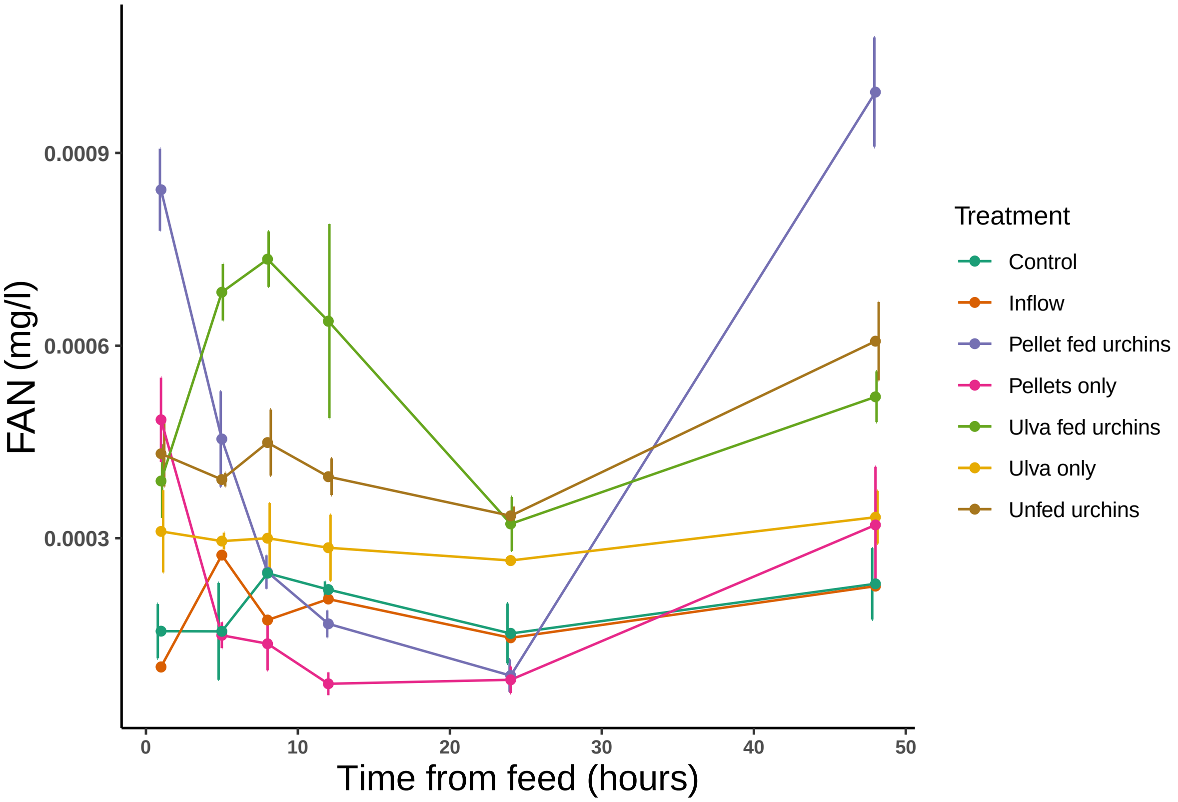


Fig. 4.3 Free ammonia nitrogen (FAN) values over time recorded from the inflow and outflow of tanks stocked with or without *Tripneustes gratilla* and fed either fresh *Ulva lacinulata*, formulated feed (pellets) or no feed (unfed). Control samples represent tanks without urchins and feed. Vertical bars represent standard deviation, and the data points represent the means. Shaded blocks show which periods were night time**.**

4.3.2 Nitrate

The nitrate concentration of inflowing water was on average 0.64±0.03 mg/l. A repeated measure ANOVA demonstrated that the main effects and all the interactions between presence of urchins, feed type and time from feed were all significant ($F_{(1/2/5/10,\ 78)} > 3.303$, $p < 0.009$). The lack of significance between the inflow and the control ($p = 0.449$) suggests the tank itself did not influence the NO_3 concentration. When urchins were fed *Ulva*, an average nitrate concentration of 0.83±0.127 mg/l was recorded in the effluent. This is 1.3 times higher than the concentration of nitrate in the inflowing water and significantly greater than all other treatments ($p < 0.001$, Fig. 4.4). Tanks without urchins, but provided with pellets, had significantly lower nitrate concentrations than all other treatments ($p > 0.046$, Fig. 4.4). When pellets were provided to urchins, the NO_3 values did not differ significantly from the control ($p = 0.081$, Fig. 4.4), but this treatment did have significantly reduced nitrate concentrations relative to the inflow ($p = 0.022$, Fig. 4.4). Further evidence of the interaction between feed and presence of urchins was displayed by the significant difference in nitrate concentration of the outflow of tanks containing pellet-fed urchins to those tanks with pellet only ($p = 0.0457$, Fig. 4.4) as well as *Ulva*-fed urchins and *Ulva* only ($p < 0.001$). Unlike TAN, there is no indication of accumulation of NO_3 over time (Fig. 4.5)

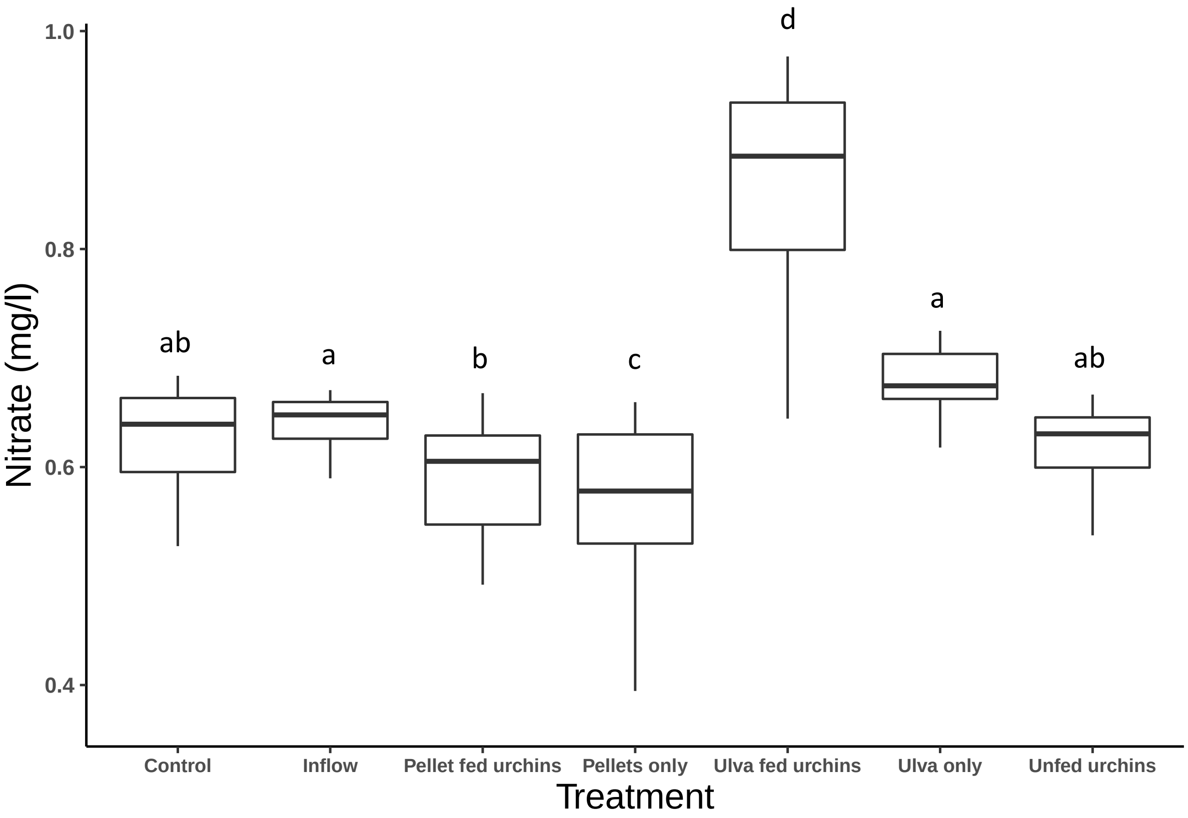


Fig. 4. 4 The influence of urchins and their feed on nitrate concentrations with results pooled over the 48 hour experimental period. The dots represent outliers, and the different letters denote significant differences from a Student–Newman–Keuls (SNK) test.

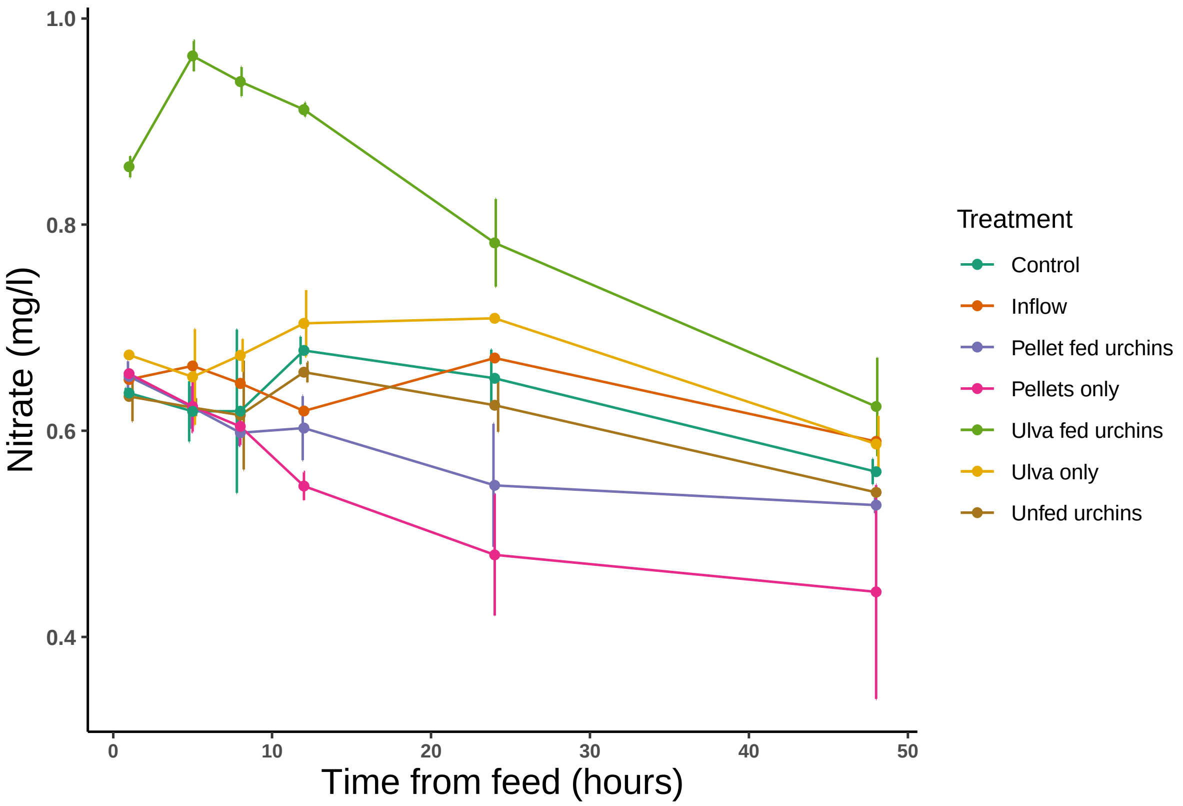


Fig. 4.5. The effect of *Tripneustes gratilla*, pellets and *Ulva* on nitrate concentrations over the 48-hour sampling period. The data points represent the means, the vertical bars indicate the standard deviation between replicates (tanks) and the shaded blocks represent night time.

4.3.3 Nitrite

The average NO_2 of the inflowing water was 0.022±0.002 mg/l. While time and feed type have significant main effects on NO_2 ($F_{(2/5, 78)} > 32.464$, $p < 0.001$), the presence of urchins did not directly influence NO_2 levels ($F_{(1, 78)} = 0.028$, $p = 0.867$). The interaction effect of feed type and presence of urchin, however, was significant ($F_{(2, 78)} = 31.649$, $p < 0.001$), suggesting the influence feed type has on NO_2 concentration is dependent on whether or not there are urchins present in the system. This is apparent in tanks provided with *Ulva*, where those with urchins had significantly higher NO_2 levels in the outflow than those tanks without urchins ($p = 0.006$, Fig. 4.6). The relationships between treatments on nitrite were very similar to those of nitrate, but the nitrite was an order of magnitude lower than that of nitrate (Figs. 4.5 and 4.6). The nitrite concentration of water exiting the pellet-fed tanks with and without urchins did not differ significantly ($p = 0.166$). The minimum nitrite value was 0.009 mg/l, observed 24 hours after pellets were fed to the urchins (Fig. 4.7). This was 57.42% lower than the inflow at that time.

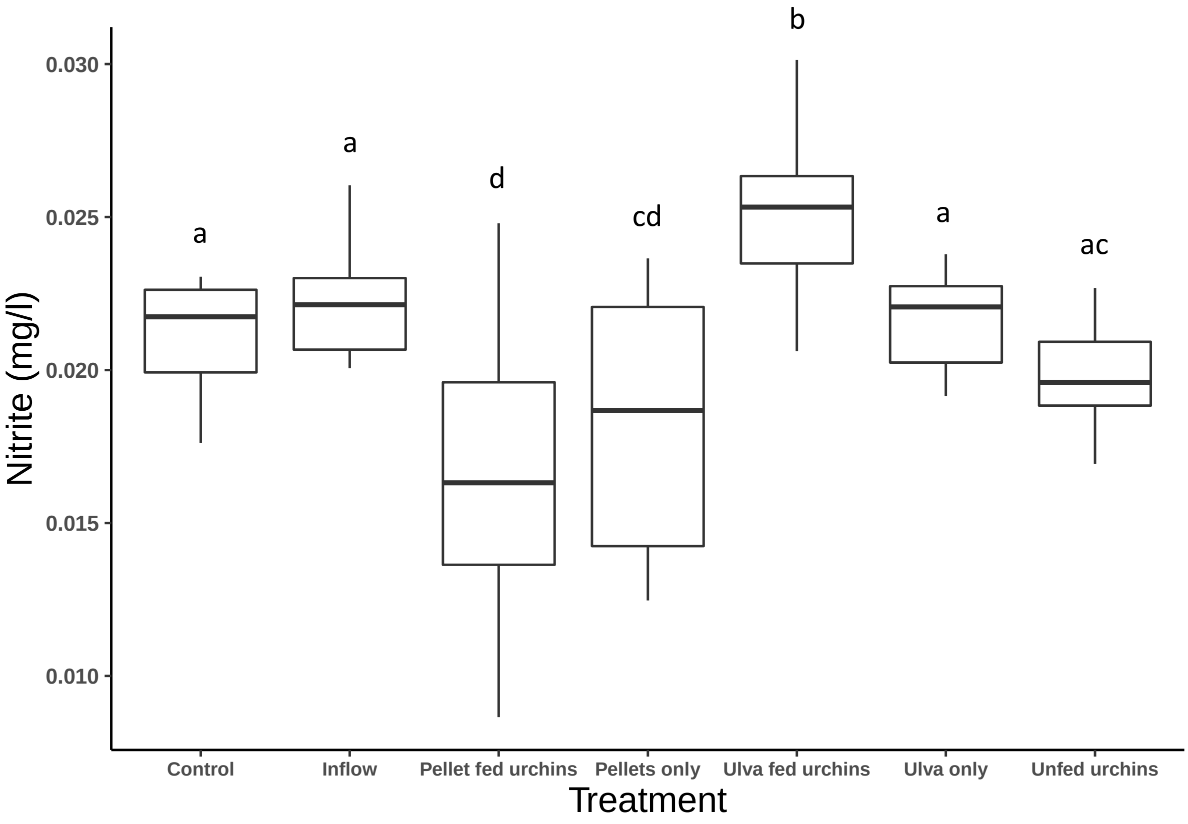


Fig. 4.6. Nitrite concentration compared in the inflow and outflow of tanks treated to various combination of *Tripneustes gratilla* presence/absence and feed types. All samples taken over the 48-hour period were pooled within treatments for this graph. The letters correspond with significance deducted from a Student–Newman–Keuls (SNK) test, where the same letters indicate no significant differences between treatments.

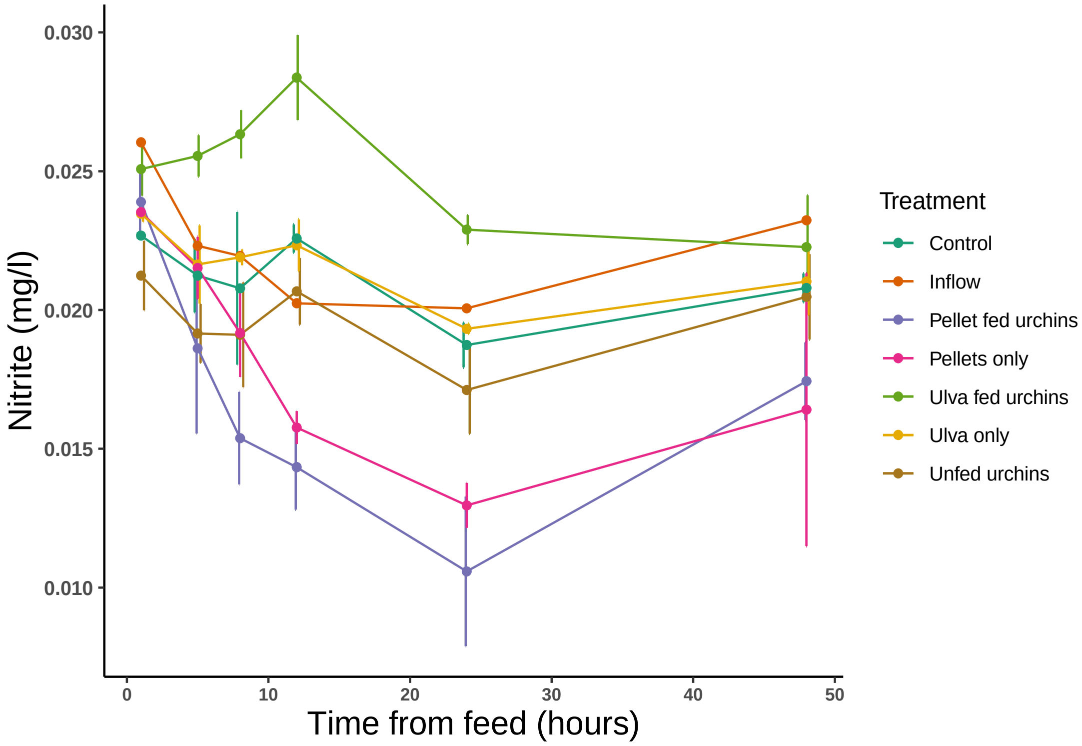


Fig. 4.7. The effect of *Tripneustes gratilla* and their feed on nitrite concentrations over the sampling period. The data points represent the mean value of the treatments at the specific time interval, the vertical lines indicate the standard deviation observed between replicates and the shaded blocks represent night time.

4.3.4 Phosphate

The average concentration of phosphate in the effluent of tanks with or without urchins an hour after pellets were introduced into the system was 0.05 mg/l, which is more than 14 times greater than the inflowing water (Fig. 4.9). There were statistically significant main effects and interactions between presence of urchin and time ($F_{(1/5,\,24)} > 11.641$, $p < 0.001$) for the treatments with pellets. Phosphate levels were significantly, but not severely, higher in the outflow of tanks in which urchins were fed pellets compared to those tanks supplied with pellets that did not contain urchins ($F_{(1,\,24)} = 16.026$, $p < 0.001$). The release of phosphate by pellets was substantial but declined rapidly over the first eight hours (Fig. 4.9). Following this, the phosphate levels remained at a relatively stable concentration of approximately 0. 0045 mg/l. This concentration is still greater than the average values of all other treatments (Fig. 4.8). The lack of a significant difference ($p = 0.238$, Fig. 4.8) in phosphate concentrations between the inflowing water and the outflow of the control tank (that had no urchins and no feed) implies that the tank itself has a negligible influence on phosphate concentrations. The tanks housing unfed urchins and those with only *Ulva* (no urchins) had significantly higher phosphate concentrations than the inflow ($p < 0.002$). The tanks with urchins fed *Ulva* had significantly more phosphate in their outflow than the other non-pellet treatments ($p < 0.001$). For these treatments (not including pellets), the main effects of feed type and presence of urchins and the interaction between these two factors had a significant effect on phosphate levels ($F_{(1,47)} > 18.30$, $p < 0.001$). The time from feeding did not have a significant influence ($F_{(5,47)} = 1.863$, $p = 0.119$), suggesting that the phosphate released is fairly constant over time.

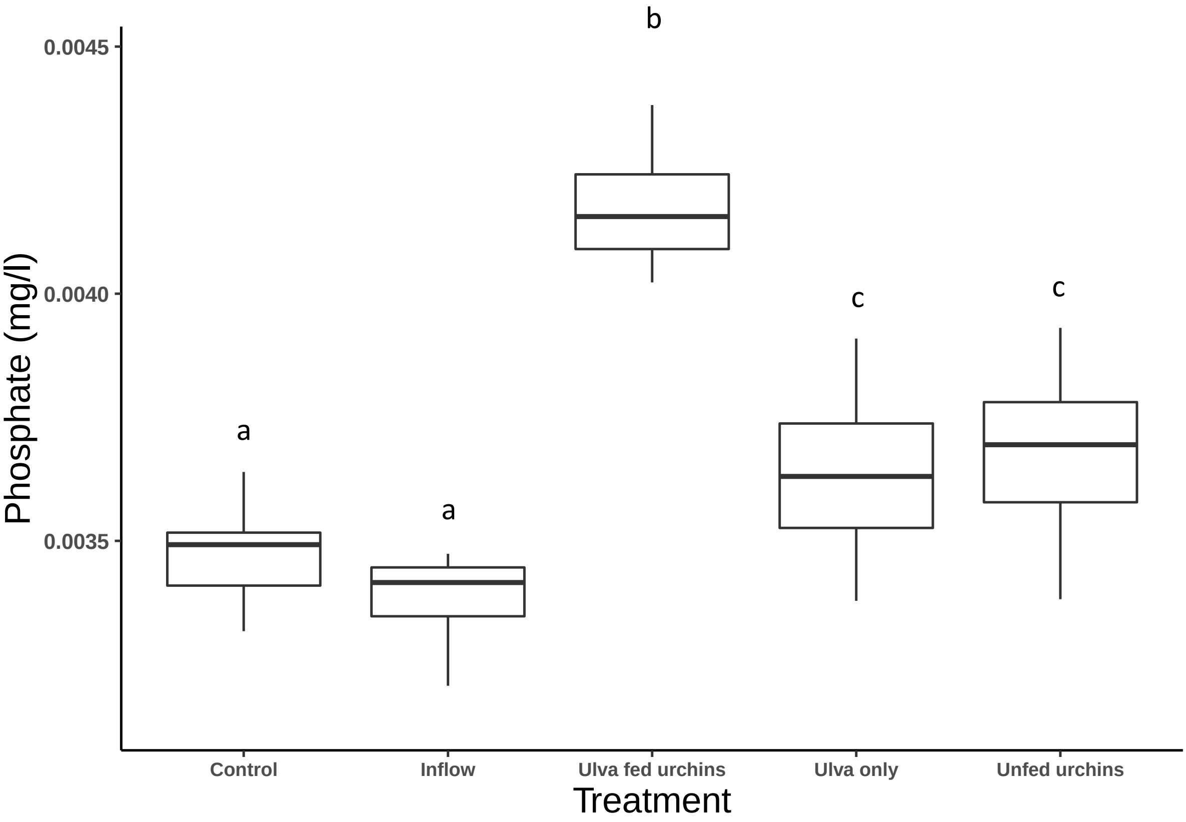


Fig. 4.8. The influence the feed and presence treatments had on phosphate levels with results pooled over the 48 hour experimental period. The opposing letters indicate significant differences based on a Student–Newman–Keuls (SNK) test. The treatments provided with pellets could not be included here as their phosphate levels were on a different order magnitude.

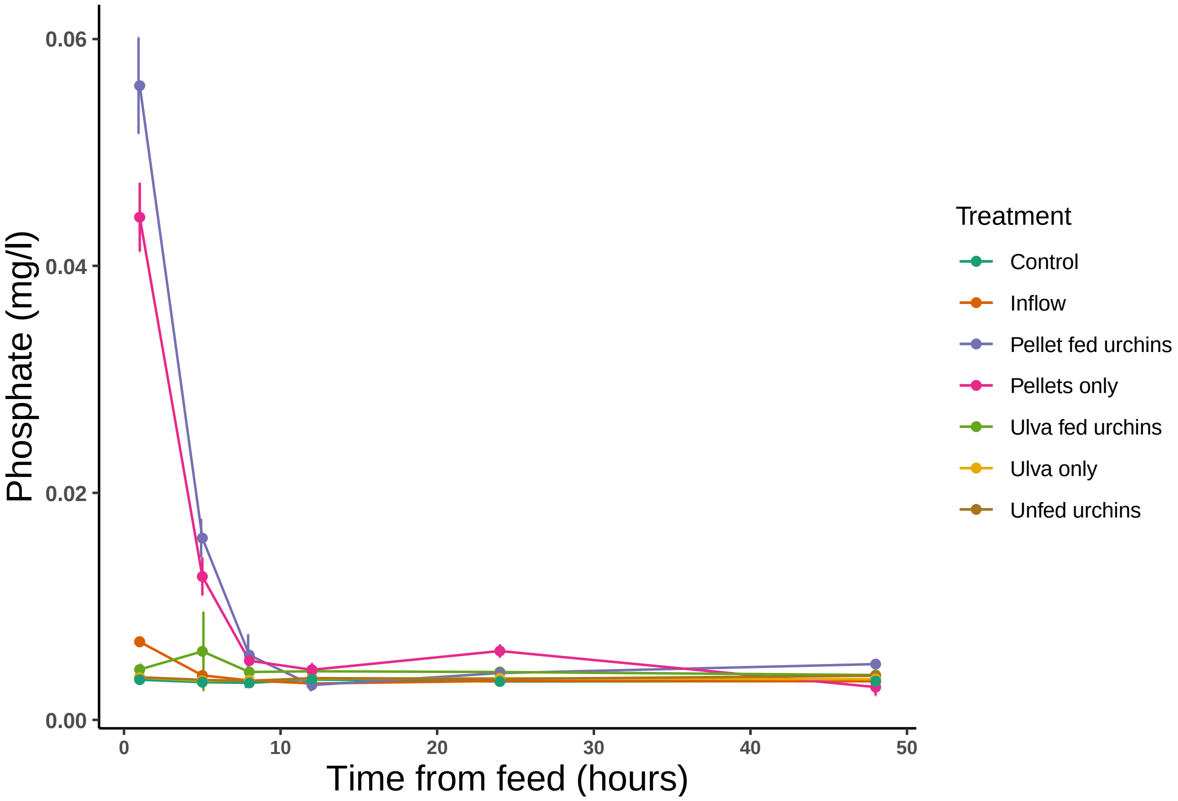


Fig. 4.9. The phosphate levels observed over the 48-hour period between the various treatments. The data points are the means, the vertical bars indicate the standard deviation and the shaded blocks represent night time.

4.3.5 Total Suspended Solids (TSS)

The average TSS of the inflowing water was 0.152±0.009 mg/l which was greater than the average outflow 0.142±0.018mg/l of tanks across all treatments. It appears that as water flows through the tanks the level of TSS is reduced, because the mean of the control is lower than the inflow (Fig. 4.10). Yet, this was not supported statistically, where no significant differences were detected for main or interacting effects ($F_{(1/2,\ 14)} > 1.809$, $p > 0.2$). Only one sample time was retrieved during this trial. As such, sample sizes were small (n = 3) and the effects of time on TSS is unknown.

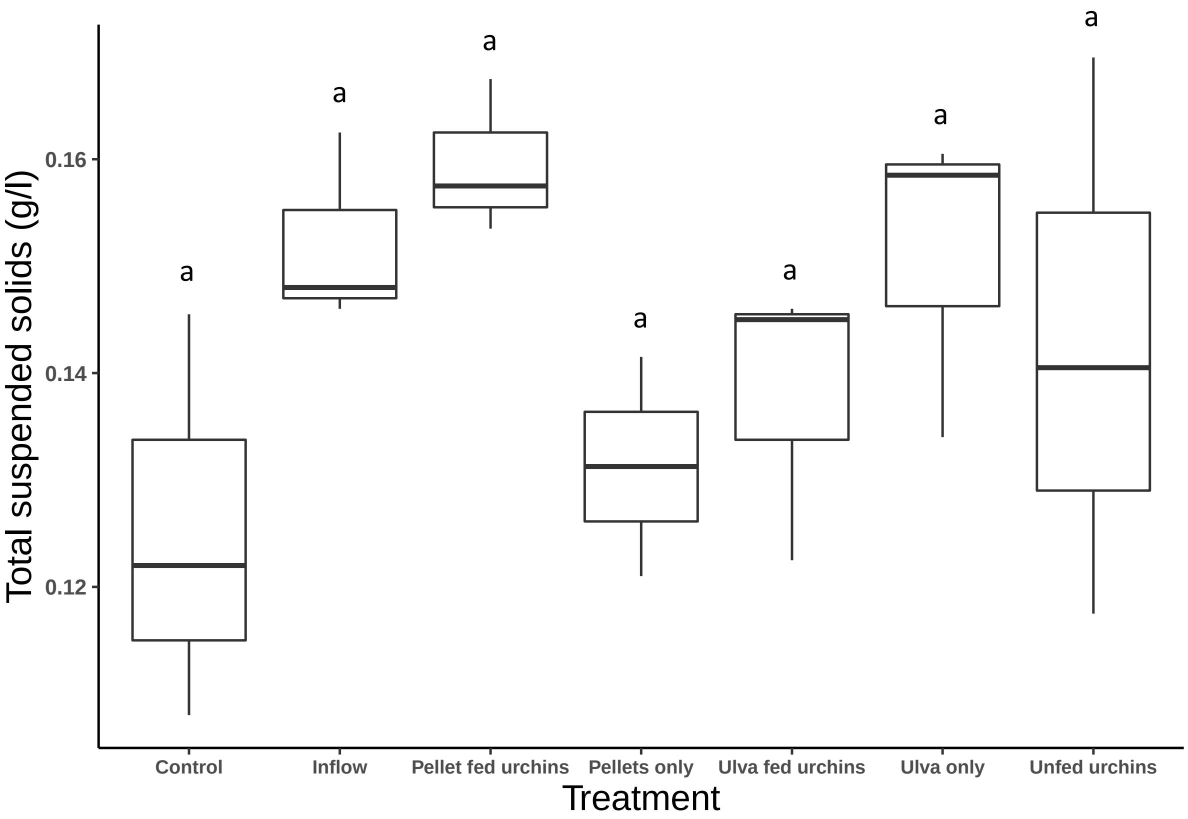


Fig. 4.10. The total suspended solids produced from the various treatments and inflow only from a single time interval. The corresponding letters indicate there was no evidence of significant differences between treatments resulting from a pairwise comparison.

4.3.6 pH

The mean pH of the inflow was 8.145±0.049. There was evidence that the main effects; urchin presence, feed type and time from feed, significantly influenced the pH ($F_{(1/2/5, 78)} > 9.221$, $p < 0.001$). There were also significant interactions between both feed and the presence of urchins ($F_{(2, 78)} = 41.274$, $p < 0.001$), as well as feed type and time from feed ($F_{(10, 78)} = 3.284$, $p < 0.001$). The inflow did not differ significantly from the control, but evidence was marginal ($p = 0.056$, Fig. 4.11). The control tank did not differ significantly from the tank containing *Ulva* without urchins ($p = 0.492$, Fig. 4.11). The tanks where urchins were fed pellets had significantly lower pH values than all other treatments ($p < 0.006$, Fig. 4.11), with the minimum recorded observation of 7.75. The *Ulva*-fed urchins had the second lowest pH values, with a mean pH of 7.903 (Fig. 4.12).

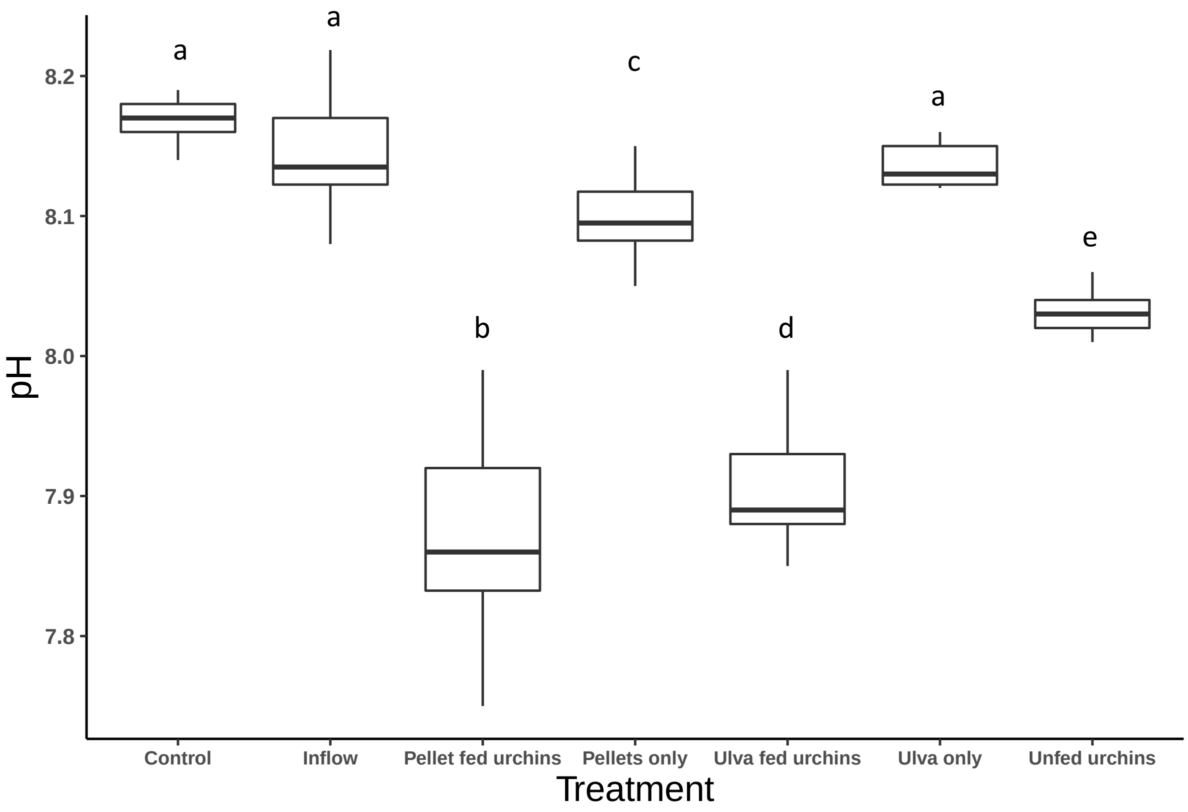


Fig. 4.11. The effect of *Tripneustes gratilla* and various feed types on the pH of the outflowing water, pooled over a 48-hour period. Letters indicate significant differences based on a Student–Newman–Keuls (SNK) test.

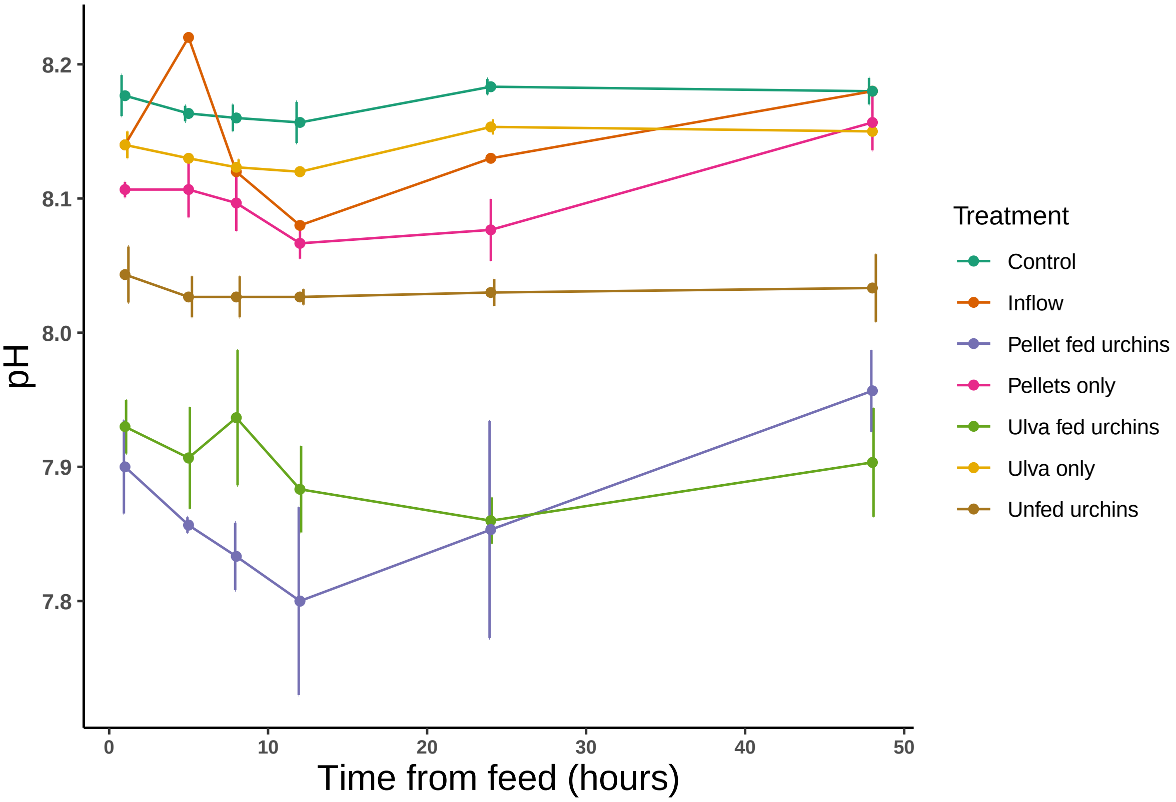


Fig. 4.12. The pH between various treatments over time. The straight lines, especially during the 12, 24 and 48-hour sampling intervals, are unlikely to be highly accurate, as diurnal fluctuations are likely. Data points represent the means, the vertical bars indicate the standard deviation and the shaded sections represent night time.

4.3.7 Alkalinity

The mean alkalinity, expressed as the concentration of calcium carbonate ions, at the inflow was 134.5±0.707 mg/l. The outflow increased on average by approximately 5mg/l across all treatments, including the control (Fig. 4.13). The only significant effect on alkalinity was time from feed ($F_{(1, 24)}$ = 7.052, p = 0.014). In this case, the calcium carbonate concentration 12 hours after feeding, at 6 pm, was on average 137.706±2.845 mg/l, while 24 hours after feeding saw an average calcium carbonate concentration of 140.473±3.486 mg/l.

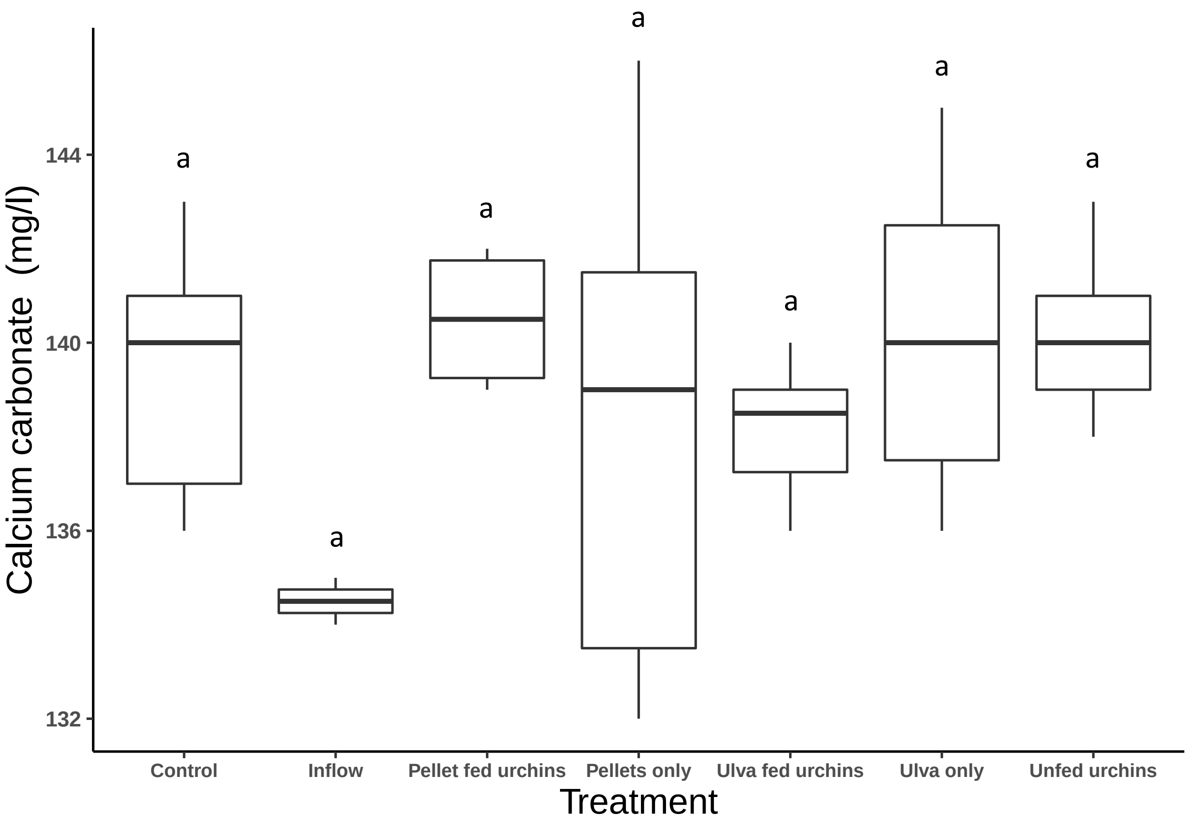


Fig. 4.13. The alkalinity, expressed as calcium carbonate, compared in the inflow and outflow of tanks treated to various combination of T*ripneustes gratilla* presence/absence and feed types over a 48 hour period. The corresponding letters indicate that there were no significant differences between treatments.

4.3.8 Dissolved carbon dioxide

The mean $p\text{CO}_2$ of the inflow was 189.001 µATM, while the maximum value was 525.117 µATM observed in the pellet-fed treatment. While the time when the samples were taken did not significantly influence the dissolved carbon dioxide content of the water ($F_{(1,\ 24)}$ = 0.447, p = 0.510), feed treatment, presence of urchins and their interaction had a significant effect ($F_{(1/2,\ 24)}$ > 11.081, p < 0.001). While the pellet-fed urchins had a greater mean $p\text{CO}_2$ than the *Ulva*-fed urchins, the difference between these treatments was found to not be significantly different (p = 0.121). The dissolved CO_2 concentration found in the outflow of the tanks containing no urchins or feed (control) did differ significantly from the unfed urchin treatment (p = 0.033).

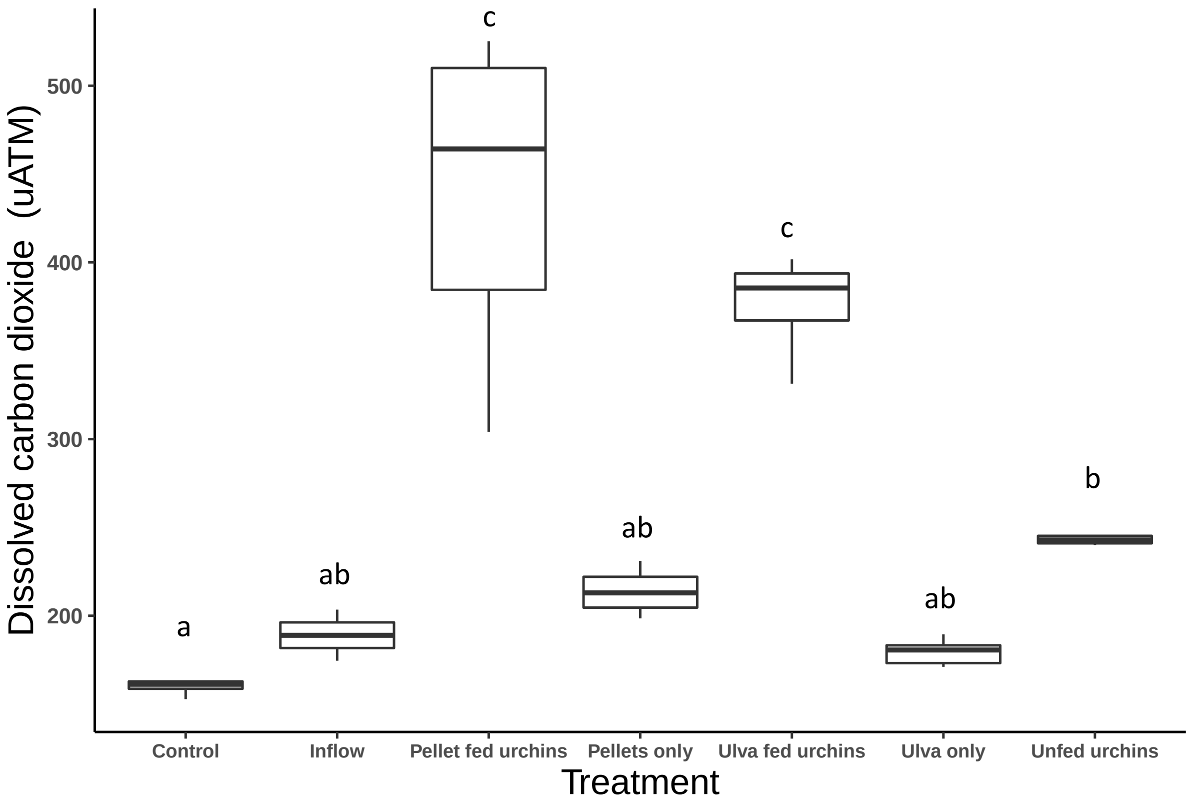


Fig. 4.16. The influence of Tripneustes *gratilla*, *Ulva lacinulata* and a formulated feed on dissolved carbon dioxide (*p*CO2) concentration over a 48 hour period. Letters denote significance based on the results of a Student–Newman–Keuls (SNK) test.

4.4 Discussion

The influence and interactions of feed type, urchin presence and time from being fed on water chemistry can be clearly observed by the total ammonia nitrogen (TAN) emissions. The tanks provided with formulated feed (regardless of urchin presence) demonstrate a TAN spike an hour after the pellets were added. This is a regular occurrence when formulated feed is used in aquaculture and is known as nitrogen leaching (Piedecausa *et al.*, 2010). The amount of nitrogen leached from the pellets is significantly lower in tanks without urchins compared to the tanks with urchins. This reflects the urchins breaking up the pellets while feeding, therefore increasing the surface area of the pellets, which allows for greater leaching. The pellet-only tanks (with pellets and without urchins) demonstrated a consistent TAN concertation after this initial spike for the duration of the experiment. The pellet-fed treatment had a similar decline in TAN emissions after the initial spike. However, at the final sampling interval, 48 hours after the feed was supplied, the TAN concentration in the effluent was on par with the initial TAN spike. This last spike is highly correlated with sludge accumulation (and its influence on DIN concentrations) and gut transit times of *T. gratilla* (Hargreaves, 1998; Klinger *et al.*, 1994; Lewis, 1967; Stewart *et al.*, 2006; Vaïtilingon *et* *al.*, 2003). This finding indicates the importance of regular sludge removal. While the average TAN emission did not differ significantly between the pellet-fed urchins and *Ulva*-fed urchins, the dynamics of the emissions were very different. Unlike the aforementioned spikes of the pellet-fed urchins, the *Ulva*-fed urchins demonstrated a consistent accumulation, followed by a decline of TAN emissions over the 24-hour period. This corresponds with feeding observations; where not all urchins had begun feeding at the first sampling period, then, for the following three sampling intervals, were feeding voraciously until all feed was consumed at 24 hours. This implies the TAN emissions were likely from ammonia/urea release from *T. gratilla* and/or *T. gratilla* shredding the *Ulva,* thereby releasing its internal nitrogen (Sabourin and Stickle, 1981; Zhang and Wang, 2017). The unfed urchins demonstrated a low but consistent TAN emission (average of 0.01 mg/l), which was likely the waste products of the baseline metabolic processes. The *Ulva*-only treatment (tanks without urchins but with *Ulva*) and control tanks (no feed or urchins) did not have a significantly different DIN concentration to the inflow. Therefore, there is no evidence that the tank itself (and its microbial community etc) and the *Ulva* alone has a significant influence of DIN emissions within these conditions. This is likely due to the low light levels, which are suited to *T. gratilla* culture, as discussed in the methods.

The TAN emissions observed here largely correspond with those found in other urchin studies. The daily net production of TAN from this trial (calculated from the integral of the TAN emission model described in Chapter 5) was approximately 0.391 and 0.287 mg TAN individual^{-1}. day^{-1} for the *Ulva*- and pellet-fed *T. gratilla* respectively (average individual mass of 142 g each). These values are an order of magnitude lower than the values reported for *T. gratilla* in the two other nitrogen emission studies (Dy and Yap, 2000; Koike *et al.*, 1987b), yet correspond with the approximate average emission of adult urchins reported across various studies, species and sizes, which was 0.234mg TAN.individual^{-1}.day^{-1} (Arafa *et al.*, 2006; Asnicar *et al.*, 2021; Brockington and Peck, 2001; Hill and Lawrence, 2006; Wai and Williams, 2005). The reason for the previously reported TAN emission of *T. gratilla* being considerably higher than this study and the other urchin studies listed here could be linked to sample collection methodology, as previously described. Yet, the primary point is that the TAN levels observed in this study are likely reliable as they resemble those seen in other urchin studies.

The TAN concentrations recorded in this study were relatively low (average 0.01 mg/l) and do not appear to have an immediate threat to production or the environment. For context in aquaculture, TAN concentrations exceeding 25 mg/l have been observed (Barnharst *et al.*, 2018) and the

Aquaculture Stewardship Council offers their certificate to abalone farms with TAN below 0.774 mg.l^{-1} (ASC, 2012) amongst other criteria. The dissolved inorganic nitrogen (DIN) levels observed during these trials were well below the levels that affect urchins, as indicated in other literature (Basuyaux and Mathieu, 1999; Lawrence *et al.*, 2003; Siikavuopio *et al.*, 2004a, 2004b). There is, however, some indication of potential risks if conditional parameters are changed. The TAN and FAN levels reached a maximum of 0.031 mg/l and 0.001 mg/l respectively and occurred when pellets were fed to urchins. The maximum TAN level occurred an hour after feeding, while the maximum FAN level occurred 48 hours after feeding due to the higher pH levels during this period. The maximum FAN levels observed in this study were well below the level (0.016 mg/l) that reduced gonad yield of *S. droebachiensis* by approximately 18%, but did not significantly increase mortality compared to a control treatment (Siikavuopio *et al.*, 2004b). This does not though, suggest that TAN is not a potential threat to the production of *T. gratilla*. It is important to note that the tanks had been siphoned (cleared of sludge) 12 hours prior to feeding and the conditional parameters applied for this study were conservative. This applies in particular to flow rates (approximately one turnover per hour) and stocking densities (approximately 60kg.m^{-3}). A plausible (but not advised) farm scenario could be a doubled stocking density, the flow rate reduced to 0.25 turnovers per hour, and urchins fed 48 hours after the previous feeding without having been siphoned. Assuming the influence of these factors on TAN concentration is linear and compounding, this scenario could result in FAN levels of approximately 0.016mg/l. While this may not result in immediate mortality (Siikavuopio *et al.*, 2004b), it could reduce production as a negative linear relationship between production and TAN concentration has been demonstrated for various aquatic organisms (Colt and Tchobanoglous, 1978; Huchette *et al.*, 2003). Specific ammonia exposure trial and nitrogen modelling for *T. gratilla* are, however, required to confirm this suspicion. As flow rates and stocking densities are likely to be less flexible parameters for economic reasons, this finding suggests the importance of regular sludge removal, especially prior to feeding, to prevent the accumulation of TAN. The nitrite levels reached a maximum of 0.03 mg/l 12 hours after *Ulva* was fed and the maximum nitrite concentration for the inflow was 0.026 mg/l. While certain treatments had significantly different values, this suggests urchins and their feed did not have a strong influence on nitrite concentrations that were also well below the 'low' levels of 0.5 mg/l, which reduced gonad mass of S. *droebachiensis* (Siikavuopio *et al.*, 2004a). Similarly, with regards to nitrate, only when *Ulva* was fed to the urchins was its concentration significantly but not considerably greater than the inflowing water. This implies a majority of the dissolved nitrogen emissions from *T. gratilla* is TAN, which is in agreement with other studies (Sabourin and Stickle, 1981).

Formulated feed in aquaculture is generally associated with high nitrogen emissions (Piedecausa *et al.*, 2010). Thus, it was unanticipated to find that for specific time intervals the inflowing water had greater concentrations of all DIN species than the outflow from all tanks containing pellets. This was most apparent 24 hours post feeding for nitrite, where the nitrite concentration at the inflow was half of what is at the outflow. This could even be interpreted as the pellets “filtering” 57.42% of the nitrite of the system. This may be the result of absorption by activated carbon derived from the ingredients found in the pellet, possibly the wheat bran (Zarabi and Jalali, 2018) or maize. Rice husks have been observed to absorb nearly 50% of ammonium from an aqueous solution (Zhu *et al.*, 2012). FAN (NH_4^+) is known to be loosely attracted to (or absorbed by) negatively charged cation exchange sites (Hargreaves, 1998), which are likely found on such organic matter. This absorption does not result in its complete removal from the system and it may return to the water column once the feed is consumed and excreted. As such, it will not have a strong influence on the total nitrogen balance of a system unless the pellets are not consumed and/or removed. Another possibility of this reduction of DIN may be due to a relationship between the microbial community and the pellets. The microbe population could be enhanced by the high initial levels of phosphates and then limited by nitrogen concentrations. A microbiome assay may verify this microbiome role, while evidence of absorption of DIN by pellets could be provided if the experiment was repeated in sterile water.

The phosphate that leached from the pellets was remarkably high; 14 times greater than concentrations in the inflowing water. This does not though, appear to be of direct concern for urchin production as the maximum phosphate levels observed in this study were still an order of magnitude lower than values that reduced production of *Lytechinus variegatus* (Böttger *et al.*, 2001). Similarly, this indicates the phosphate emission from *T. gratilla* production is unlikely to have environmental impacts. Assuming all the phosphorus is in the form of phosphate, the average concentration observed in this study was approximately 0. 002 mg/l while the maximum phosphate threshold as defined in the Aquaculture Stewardship Council Salmon Standard is 0.02 mg/l (ASC, 2017), and not mentioned in their Abalone Standard (ASC, 2012).

Across all treatments, the mean Total Suspended Solids (TSS) was 0.142 mg/l. This is considerably below the 5 mg/l threshold in the abalone standard of the Aquaculture Stewardship Council (ASC, 2012). Currently, there is no urchin standard. It is important to note that the outflow of these tanks was at the surface. If the outflow was from the bottom of the tank, the TSS concentrations may have been greater. The outflow from the surface was chosen as it reflects what most aquaculture facilities use in the region because of its simpler design requirements. There were no significant differences in TSS between treatments. While there is a lack of statistical evidence, it appears the control had lower TSS levels relative to the inflow. This suggests it acts as a settlement tank, even with the high levels of aeration. The tank with pellets did have significantly greater TSS than the other treatments, implying that the stability of the pellets is adequate.

Besides the control and *Ulva*-only, all treatments significantly reduced pH values compared to the inflow. In finfish aquaculture, reduced pH is frequently considered beneficial as it converts FAN into less harmful ammonium. Conversely, there is strong evidence that in the culture of calcifying marine invertebrates (and *T. gratilla* specifically) lower pH has a strong negative correlation with production (Mos *et al.*, 2016; Shpigel and Erez, 2020). The diminished pH from the pellet-only treatments is likely due to the greater level of nitrification, resulting in an increase in hydrogen ions (Boyd *et al.*, 2016). The reduction of pH in the tanks containing urchins was likely the result of elevated CO_2 from respiration, assimilation of carbonates for calcification of shells, nitrification and internal balancing of pH (Boyd *et al.*, 2016; Mos *et al.*, 2016). The minimum pH value observed here (7.75) was comparable to values reported in similar stocking densities and flow rates by Mos *et al.*(2016). The reduction in pH related to the feed of pellets and *Ulva* seems to be somewhat temporary, where the lowest pH values are observed between 12 and 24 hours after feeding and then appear to begin returning to ambient conditions. The inflowing water exhibited a diurnal cycle, with higher levels during the day. This was expected because the intake for the Marine Research Aquarium, where the experiments were conducted, is located within a kelp (*Ecklonia maxima*) forest. The control tank did not result in significantly different pH values but does appear to stabilise the pH compared to that of the inflow, the reason for this is unclear. The pH observed in the unfed urchin treatment was consistent over time, suggesting the metabolism of *T. gratilla* may not fluctuate diurnally when not provided with feed.

This study did not provide clear evidence that *T. gratilla* in commercial scenarios will immediately influence alkalinity, although there is strong evidence from elsewhere that it will be reduced over a greater time period, such as weeks (Mos *et al.*, 2016). The alkalinity levels observed in this study are unlikely to influence the production of *T. gratilla* as the values observed here were near ambient (120 mg/l $CaCO_3$; Boyd *et al.*, 2016). Only when $CaCO_3$ falls below 20 mg/l does the lack of buffering potential generally become problematic with regards to FAN concentration (Hariyadi *et al.*, 1994). The higher dissolved carbon dioxide (pCO_2) in the fed urchin tanks was likely primarily derived from greater respiration because it was significantly higher than the treatments with unfed urchins and the feed without urchins. Elevated concentrations of $p\text{CO}_2$ will reduce the production of urchins as it will disturb their internal pH balance and may result in hypercapnia (Byrne and Hernández, 2020). The maximum $p\text{CO}_2$ (525 mg/l) for the pellet-fed urchins was very near (within 50 mg/l) to exceeding the values that have been found to significantly adversely impact *T. gratilla* (Brennand *et al.*, 2010; Mos *et al.*, 2016) and other urchin species (Albright *et al.*, 2012; O'Donnell *et al.*, 2010, 2009; Sunday *et al.*, 2011). It is further noteworthy that the $p\text{CO}_2$ values observed for the *Ulva*-fed urchins were not significantly lower than those of the pellet-fed urchins. As this parameter is mostly near critical levels, this study suggests this will most likely reduce the production of *T. gratilla* in a commercial setting. This notion is widely supported (Byrne *et al.*, 2013; Dubois, 2014; Mos *et al.*, 2016; Shpigel and Erez, 2020; Stumpp *et al.*, 2012). Furthermore, this threat will be exacerbated by ocean acidification (Lester, 2021). Specific research is required to determine effective amelioration methods of carbon chemistry in *T. gratilla* production. However, there is evidence that integration with *Ulva* (or other algae species) would greatly reduce $p\text{CO}_2$ and increase pH thus increasing *T. gratilla* production (Lester, 2021).

This experiment simultaneously provides qualitative and quantitative data, which allows for the comprehension and prediction of the water chemistry in *T. gratilla* effluent when fed *U. lacinulata* or formulated feed (as described in Chapter 2). This provides not only foresight into how water chemistry may reduce *T. gratilla* production (due to toxicity) but also the potential environmental impact via effluent pollution and/or the filtration requirements. In overview, the nutrient emissions of *T. gratilla* were low in an aquaculture context. No parameters achieved levels that would clearly reduce *T. gratilla* production with the only exception being dissolved carbon dioxide levels, which are of concern. Generally, the water chemistry parameters were influenced clearly and reliably (low variation between replicates) by presence of urchins and feed type. This chapter provides both basic management advice, such as the necessity of regular sludge removal and gradual feeding schedules, and the data set required for modelling of water chemistry, specifically nitrogen emissions, from *T. gratilla* systems. This nitrogen emission model is the missing link for the creation of a farm-scale model simulating a *T. gratilla*-*Ulva* IMTA system.

Chapter 5: Assessing the biotechnical feasibility of *Ulva* integration with *Tripneustes gratilla* in a commercial-scale recirculating IMTA system: a farm-scale model approach

Abstract

To justify integrating *Ulva* with *Tripneustes gratilla* in a commercial-scale recirculating IMTA system, it is crucial to provide evidence that the proposed benefits of *Ulva* can be attained. The primary benefits include complete and efficient biofiltration of total ammonia nitrogen (TAN) in the urchin effluent and the production of substantial quantities of *Ulva* to feed *T. gratilla*. To investigate the feasibility of these benefits and potential urchin production projections and limitations, a farm-scale model was constructed.

To ensure the practicality and applicability of the model, a digital twin simulating the established and extensively validated abalone-*Ulva* IMTA commercial-scale systems was developed. It consisted of 42 urchin tanks (8.5 m^3 each) and a single 300 m^2 *Ulva* raceway. Various management and production parameters or submodels of both *T. gratilla* and a generic *Ulva* species were incorporated into the model based on the literature or previous findings of this thesis.

The results of the farm-scale model suggest that while the *Ulva* raceway could remove 100% of TAN emitted by *T. gratilla*, it's not efficient as a biofilter due to its excessive farm footprint. Additionally, the projected TAN emissions (average of 28 g $TAN.day^{-1}$ across various feeding scenarios) from the *T. gratilla* production system will not be sufficient to sustain the *Ulva* population where there would be a net reduction of *Ulva* biomass after approximately 22 days after being stocked. Thus, there would not be substantial *Ulva* production for *T. gratilla* feeding and therefore farm-scale model cannot justify the integration of *Ulva* with *T. gratilla* production based on the existing abalone-*Ulva* system configurations.

Nevertheless, the nitrogen provided in the urchin feed (excluding that retained by the urchins), whether it is formulated feed or *Ulva*, is likely ample for substantial *Ulva* production. Most of this nitrogen is lost in the sludge, but it could be utilised via mineralization. This approach could create a highly circular and efficient IMTA system. Therefore, this IMTA system may be biotechnically feasible if design or management adjustments were made.

The model indicates that *T. gratilla* farming using this land-based system would not be limited by ammonia toxicity, where levels would not exceed 0.018 mg/l. Additionally, there is evidence this system would be highly productive in terms of urchin production. The predicted annual whole urchin production of 323.26 t WW.ha^{-1} of water surface area (farm area occupied by urchin tanks) equates to a gonad production of 72.96 t WW.ha^{-1} if a gonad stomatic index of 22.57% can be achieved. This production is substantially greater than that reported of other high value aquaculture invertebrates. This indicates *T. gratilla* aquaculture could be a viable and lucrative industry, and this model could be used for its sustainable establishment.

5.1 Introduction

It has been suggested that *Tripneustes gratilla* is a fitting candidate for co-culture with a green macroalgae genus, *Ulva*, in a land-based, integrated multi-trophic aquaculture (IMTA) system (Cyrus, 2013; Shpigel *et al.*, 2018). In these systems the effluent from urchin tanks is directed into an *Ulva* raceway. The *Ulva* assimilates the dissolved nutrients in the urchin effluent, allowing bioremediated water to be recirculated back to the urchin tanks. It is proposed that this IMTA system will directly mitigate the two primary economic and environmental constraints of land-based aquaculture; wastewater management and feed requirements (Nobre *et al.*, 2010; Robertson-Andersson, 2003). *Ulva* has been identified as an ideal food source for *T. gratilla* for most of its lifecycle, it grows at fast rates in aquaculture effluents and may provide sufficient biofiltration to allow for a high degree of recirculation (Bolton *et al.*, 2009; Cyrus, 2013; Lawton *et al.*, 2013; Shpigel *et al.*, 2018). This means *Ulva* could be produced on site and utilised as the predominant food source for *T. gratilla*. This would reduce dependence on artificial pellet feeds, which typically contain large proportions of wild-caught fish meal and account for most of an aquaculture facility's operating expenses. Since the urchin effluent is filtered via the *Ulva* raceways, which could outperform conventional filtration systems (Copertino *et al.*, 2009), there will also be a reduction in the nutrients released into the ocean. This biofiltration allows for recirculation, which saves electricity costs incurred from pumping (Nobre *et al.*, 2010) and heating.

While there is clear evidence that recirculating IMTA systems are considerably more efficient than traditional monoculture aquaculture systems, they have not yet been widely adopted outside of Asia (Troell *et al.*, 2009). This is primarily because of biosecurity concerns, which lack evidence, from a variety of stakeholders (Bolton *et al.*, 2016) and the systems' level of complexity (Hughes and Black, 2016; Kleitou *et al.*, 2018), where, especially in the case of land-based systems, a fine balance between the various organisms cultured within the system is required. This balance is most fundamentally an equilibrium between the nutrient emissions from the fed organisms, *T. gratilla* in this case, and the nutrient assimilation of the extractive organism, *Ulva*. If the system does not achieve a balance, it will fail. For example, if the degree of biofiltration of the extractive organism, recirculation and/or flow rates are not sufficient, nutrients will accumulate in the system and could reach toxic levels. Contrary to this, if the fed organism does not provide sufficient nutrients to sustain the population of extractive organisms, then this population can crash and result in a variety of issues, from severely reduced biofiltration to eutrophication. To establish this equilibrium, certain conditions (such as flow rates, system design, biomass ratio of fed to extractive organisms etc.) need to be applied.

Determining these culture conditions are fundamental to the success of an IMTA system, but it is not straightforward. Typically, these conditions are established via a trial-and-error method, where a physical IMTA system is constructed, and conditions are applied, adapted and/or the system is reconstructed until an equilibrium is observed. This can be highly inefficient in terms of resources, time and livestock loss. A considerably more efficient, replicable and scalable method to determine optimal conditions that establish an equilibrium between species of varying trophic levels is through mathematical farm-scale simulation modelling (Chary *et al.*, 2022; Duarte *et al.*, 2003; Jiménez del Río *et al.*, 1996; Ren *et al.*, 2012). In engineered and controllable ecosystems, such as land-based recirculating aquaculture systems, simulation models can provide a wide variety of additional uses (Cacho, 1997), offering valuable insight into the functional, environmental and economic feasibility. Simulation models can also provide an opportunity for in-depth optimisation, all without having to build a physical pilot system.

The foundation of an IMTA farm-scale model should be based on the primary underlying exchanged resource (currency) between the fed and extractive organisms. In many aquaculture systems, this is nitrogen and more specifically total ammonia nitrogen (TAN). It is generally the first waste product of the fed organism to become toxic (Hargreaves, 1998). TAN, along with other dissolved nitrogen species, is also frequently the first nutrient to limit algal growth in aquaculture. A simulation of the exchange of TAN between these two (or more) reactors is a TAN mass balance model. This mass balance model indicates where production is limited (for both the fed and extractive organisms). As such, the model could be extended to create a production/system model. In turn, various parameters from other disciplines could be added to the model, such as the monetary value of urchins and the feed substitution of wild caught fish with *Ulva*, thus creating an interdisciplinary model. The availability of an interdisciplinary simulation model of *T. gratilla*-*Ulva* IMTA systems would be a valuable tool for environmental impact assessors, farmers, entrepreneurs and investors to design farms, advise management practices and conduct economic, environmental and social analyses. This could greatly assist the establishment and growth of an industry based on a recirculating urchin-*Ulva* IMTA system.

To create a nitrogen mass balance model for *Ulva* and *T. gratilla* systems, it is necessary to predict how these specific organisms affect the TAN concentration of their water in conditions like those of the intended commercial setting. There is extensive literature on how *Ulva* affects water chemistry at a commercially appropriate scale. This includes *Ulva* production and assimilation models (Hadley *et al.*, 2015; Lamprianidou *et al.*, 2015; Martins and Marques, 2002; Nobre *et al.*, 2017; Oca *et al.*, 2019; Solidoro *et al.*, 1997; Zollmann *et al.*, 2021), a dynamic energy budget model (Lavaud *et al.*, 2020) and *Ulva* nutrient assimilation and production rates (Aníbal *et al.*, 2014; Bolton *et al.*, 2009; Chatzoglou *et al.*, 2020; Copertino *et al.*, 2009; Ge *et al.*, 2019; Shpigel *et al.*, 2019, 2018). *Tripneustes gratilla* was identified as a suitable aquaculture candidate in the early 1980s (Trinidad-Roa, 1989). As such, there are decades of research and some 1 570 papers (Google scholar, 2022) dedicated to their culture. On the other hand, prior to this study, there was a gap in the literature that prevented the accurate prediction of TAN emissions from *T. gratilla* commercial aquaculture systems. Closing this gap allows the creation of a TAN mass balance model, which will enable the following objectives to be achieved.

Objectives and scope

The main goal is to determine whether a recirculating *Ulva* and urchin system is feasible from a biotechnical perspective. Specifically, the study considers whether an *Ulva* raceway could provide complete biofiltration of TAN and produce a sufficient feed for production of *T. gratilla* in a land-based recirculating IMTA system, thereby replicating the existing integrated abalone-*Ulva* production systems in South Africa. As it is not clear what feeding regime is ideal in terms of balancing nitrogen, feed requirements and gonad production, the following three feeding scenarios are simulated and compared.

Feeding scenarios:

(A) Fresh *Ulva* for the entire culture period;
(B) Formulated feed, as described in Chapter 2, for the entire culture period; or
(C) Fresh *Ulva* for the grow-out period (four months) and formulated feed for the gonad enhancement period (three months), as discussed in Chapter 2.

Explicitly, the objectives were to determine/estimate:

(1) The feasibility of the *Ulva* raceway as a biofilter for *T. gratilla* TAN emissions;
(2) The feasibility of the *Ulva* raceway as a feed source for *T. gratilla*;
(3) Potential for TAN accumulation within the *T. gratilla* system to reduce urchin production; and
(4) The production of marketable product (urchin gonad).

A broad scope was applied to enhance the applicability of this study. This means the conceptual farm described in this study has a generic and non-specified location and *Ulva* species suited to *T. gratilla* culture (as described below). Therefore, a specific environmental and bioeconomic model or lifecycle assessment cannot be made within this study and is beyond its scope. However, the model described here could be used as a foundation for these analyses. While this study provides important insight into the system's economic and environmental feasibility, it focuses on the biotechnical feasibility of this proposed *T. gratilla*-*Ulva* IMTA system.

5.2 Methods

5.2.1 Conceptual farm design

The conceptual recirculating urchin-*Ulva* system used as a basis for developing the model in this study is based on the design of one of the abalone-*Ulva* 'clusters' at Viking's Buffeljags abalone farm (Fig. 5.1). This is due to this system being validated, accessible and available (Chapter 1). The conceptual system incorporates 42 fibreglass abalone tanks (6 × 1.8 × 0.8 m, 8.5 m^3; length, width, depth, volume) to hold the urchins. The cluster also includes an *Ulva* raceway (30 × 10 × 0.5 m, 150 m^3; length, width, depth, volume), where most of the water movement is driven by a large rotating paddle wheel, and a 25 m^3 sump (settlement tank) next to the paddle raceway. This entire system would occupy approximately 1 200 m^2 of land surface area.

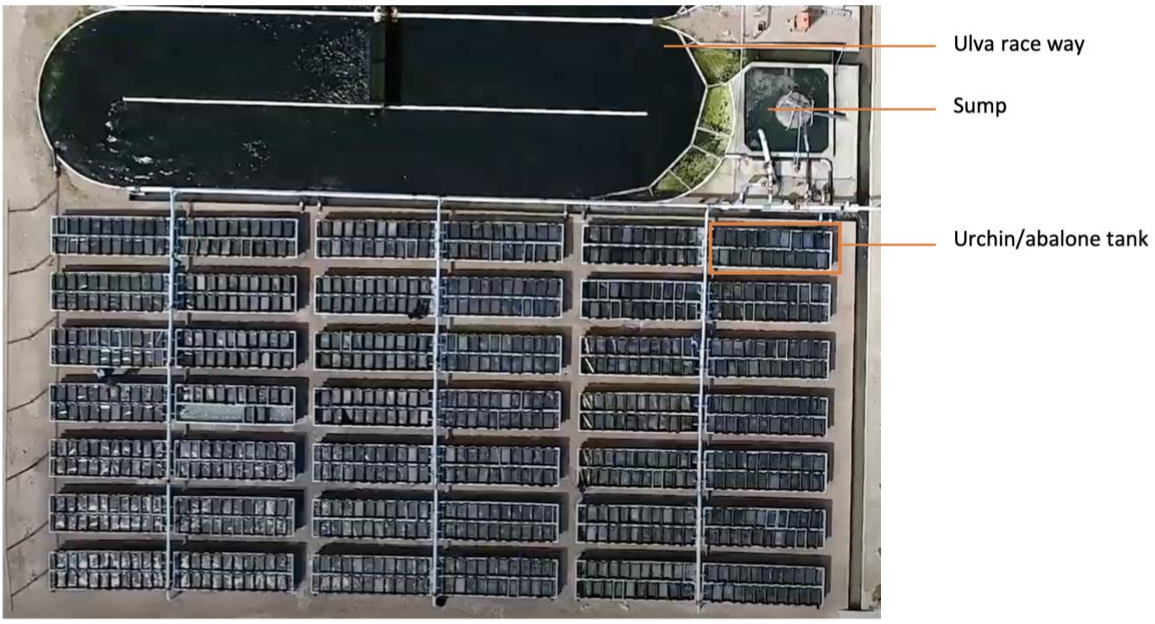


Fig. 5.1. Aerial view of one of the 28 existing abalone-*Ulva* commercial recirculating integrated multitrophic aquaculture (IMTA) systems or clusters at Viking's Buffeljags Abalone farm. This design has been replicated in this study for the conceptual *Tripneustes gratilla-Ulva* IMTA system. The labels indicate the primary components.

Each tank contains 20 baskets (0.8 x 0.5x 0.5 m; length, width, depth; justification for dimensions in discussion) made of HDPE mesh with a 6 mm pore size (Fig. 5.2). These baskets provide a surface for the urchins to attach themselves and make it possible to separate and sort them. By dividing each basket into four sections, the available internal surface area for each basket is 3 m^2, therefore providing a total surface area of 60 m^2 per tank. The outflow of each urchin tank is located at the surface of the tank. The settled particulates (sludge) are removed once every second day (Chapter 4).

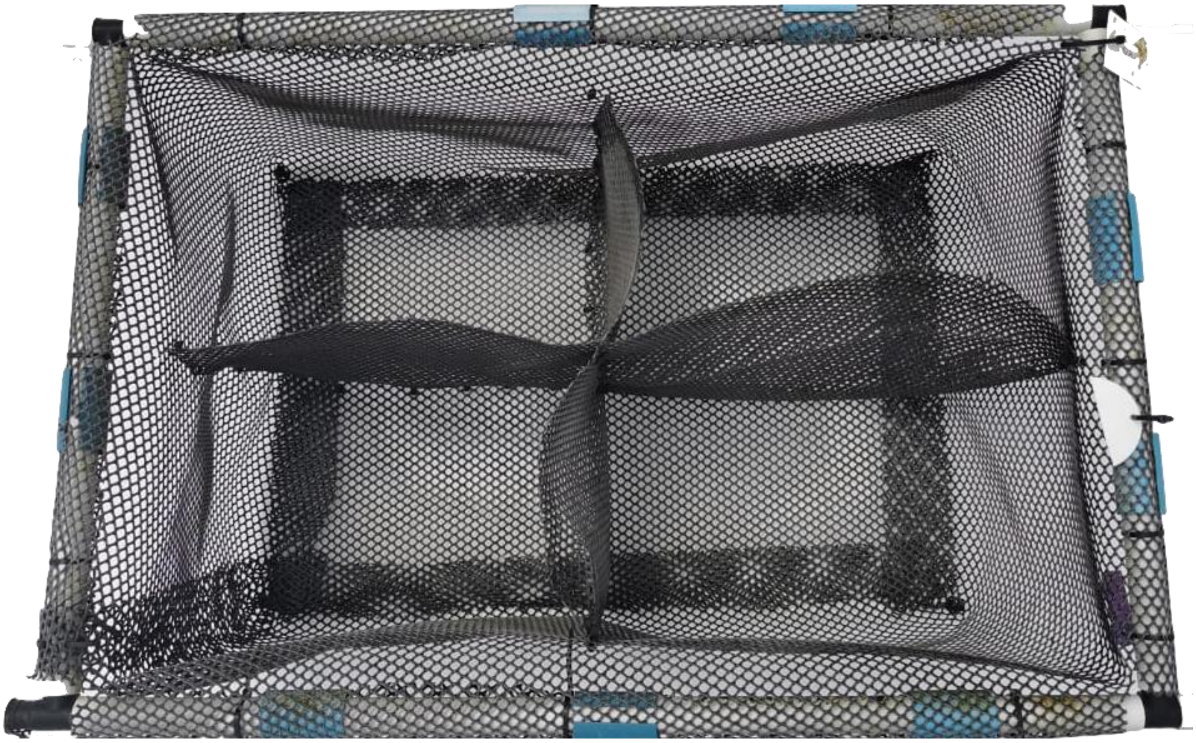


Fig. 5.2. Top-down view of an urchin basket used in the pilot and conceptual farm.

Bioremediated water from the *Ulva* raceway flows into the sump and is pumped directly into each urchin tank. Effluent water exits each urchin tank on the opposite side of the tank to the inlet water and returns directly to the *Ulva* raceway without going via any of the other urchin tanks withing the cluster. The recirculation rate (quantity of seawater added per cycle) is discussed later in this chapter.

The flow rate of each urchin tank is 4 250 l.h^{-1} (i.e., 0.5 full tank water exchanges per hour). This will result in the *Ulva* raceway receiving 178 500 l.h^{-1} (i.e., 1.19 full raceway water exchanges per hour). These rates were chosen based on the minimum values that support high urchin survival in trials on the pilot scale urchin-*Ulva* IMTA system at Viking's Buffeljags Abalone farm (unpublished) and findings from other experiments (Chapter 2, Chapter 4, Mos *et al.*, 2012).

5.2.2 Conceptual production cycle for *T. gratilla*

A production cycle of an aquaculture facility is integral to the strategic planning of various activities (such as harvesting and grading) to support growth of the cultured organisms. As there are no commercial *T. gratilla-Ulva* IMTA systems currently operational, a production cycle had to be conceptualised for this study. This cycle is recommended based on experience, findings and observations from the newly constructed urchin-*Ulva* pilot IMTA system at Buffeljags, laboratory trials at the DFFE Marine Research Aquarium, and abalone-*Ulva* IMTA farms in South Africa. These recommendations are founded on a blend of biological, economic and practical motives.

This production cycle is based on monthly rotations. Every month, market-size adults are harvested. The other urchin cohorts are graded and stocked down, which means spreading them across different baskets and tanks to ensure that they are always at optimal stocking density. A new cohort of juvenile *T. gratilla* is added once a month. Urchins are stocked into the system from hatcheries once they can be weaned on macroalgae or pellet diet and their tests measure 10 mm in diameter. Juveniles are stocked at an initial density at which, after a month, they will have grown to achieve an optimal stocking density (20% coverage as discussed in chapter 2). To prevent urchins from exceeding this limit, they are stocked down, as described above. This applies to all cohorts of urchins with every group requiring more space each month. This space is made available in the system due to monthly harvesting of market-size adults. The estimations of growth and biomass are described in Section 2.4.2.

5.2.3 Overview of farm-scale model

This model is a digital twin of the farm design (Section 5.2.1) and urchin production cycle (Section 5.2.2) with the overarching goal of assessing the biotechnical feasibility. This simplified dynamic IMTA farm-scale model is composed of two modules, representing the two species (Fig. 5.3):

1) An urchin module: an individual growth submodel combined with a simple population dynamic submodel to predict population size and biomass at cohort and

farm level. This allows for estimation of gonad production, feed requirements and TAN emissions; and

2) An *Ulva* production module: estimates *Ulva* nitrogen assimilation and biomass production, given the light levels and TAN input from the urchin system.

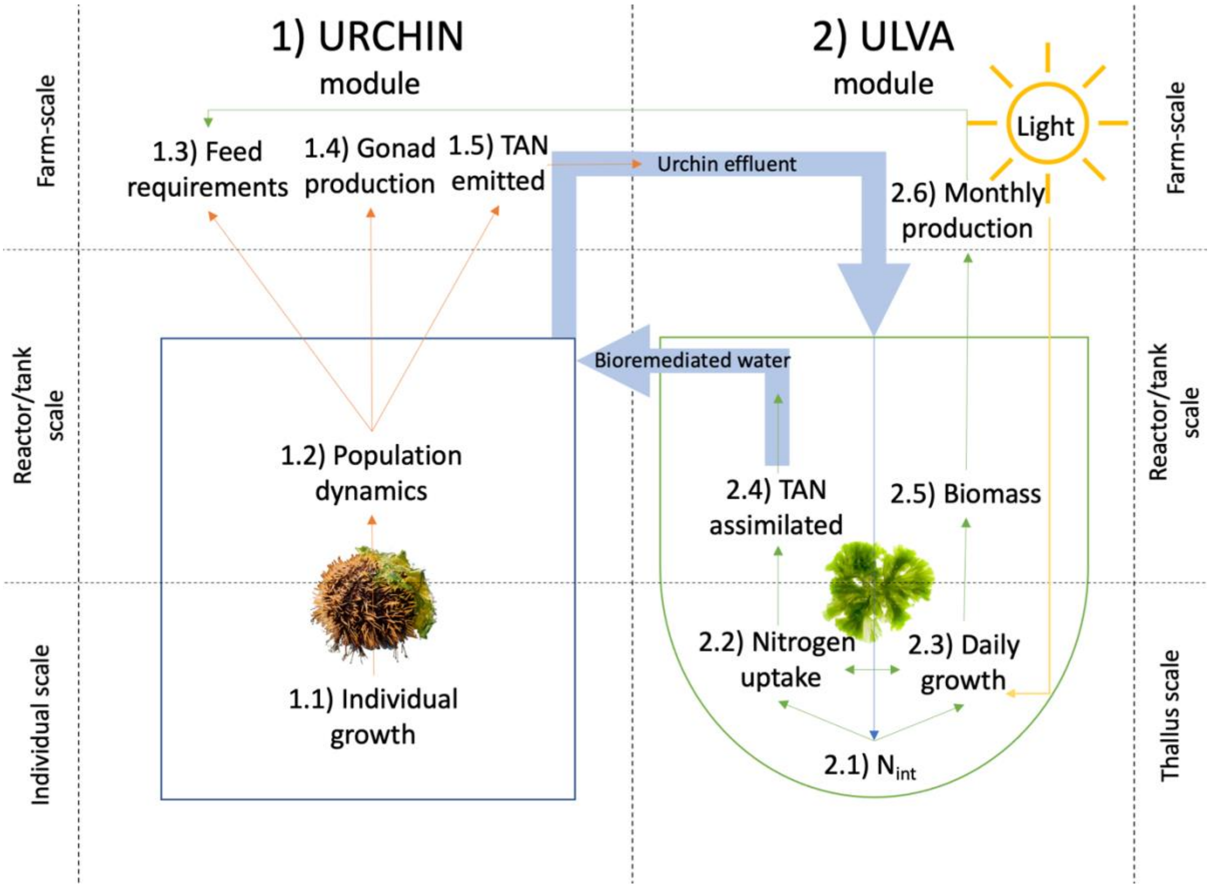


Fig. 5.3. A schematic description of the farm-scale integrated multitrophic aquaculture (IMTA) model. The urchin module (1) uses Johnson's differential growth equation (1.1) on an individual scale to estimate population dynamics (1.2), which is regulated by stocking density, available surface area and mortality/culling. This population dynamic provides biomass predictions, which are used to estimate feed requirements (1.3), gonad production (1.4) and total ammonia nitrogen (TAN) emissions (1.5). The TAN emitted from the urchins, light availability and size of the *Ulva* raceway are the primary inputs for the *Ulva* module (2). This module uses the relationship between the internal nitrogen composition of *Ulva* (N_{int}, 2.1), the nitrogen uptake (2.2) and the daily growth (2.3) to estimate the TAN assimilation (2.4) and biomass dynamics (2.5). The biomass dynamics can be used to estimate the monthly production of *Ulva* (2.6), which can be used for urchin feed.

5.2.4 Urchin module

5.2.4.1 Individual growth submodel

Johnson's differential growth equation (Ricker, 1979) was applied to predict the growth of *T. gratilla*:

$$\mathrm{dS} = \mathrm{k} * \mathrm{S_t} * \mathrm{dT}(\mathrm{InS_\infty} - \mathrm{InS_t})^2 \tag{5.1}$$

Where:

dS = urchin growth (mm)
k = constant
S_t = initial urchin diameter (mm)
dT = time (days)
S_∞ = asymptotic urchin diameter

Parameters k (2.92) and S_∞ (90) were obtained from Dafni (1992), who fed *T. gratilla* under similar aquaculture conditions to those simulated in this study, i.e. at a temperature ranging from 20.7°C to 26.7°C and urchins were fed with *Ulva lactuca* (Dafni, 1992). Johnson's differential growth equation is a semi-empirical model that uses regression to describe body mass of *T. gratilla* over a time, meaning that feed quantity and quality are not forcing variables of the model. There is evidence that feeding *T. gratilla* either fresh *Ulva* or the 20U pelleted diet (as described in Section 2.2.2) does not influence somatic growth (Cyrus *et al.*, 2015). Therefore, this same growth model was applied to the urchins, regardless of the feeding scenario.

The predictions of test diameter (S, mm) were then converted to mass (M, grams) using the following power function (Equation 5.2; Balisco, 2015):

$$\mathrm{M} = 0.07334(\frac{S}{10})^{2.6725} \tag{5.2}$$

5.2.4.2 Urchin population dynamics and biomass

The urchin population in each tank depends on the stocking density, mortality, and harvesting. The growth model suggests *T. gratilla* will take seven months to reach market size (approximately 56.26 mm test diameter) from weaning (approximately 10 mm test diameter). If a batch of weaned urchins is added each month, as described in Section 2.2, and the seven-month-old cohort is harvested, there will be a total of seven cohorts in the production system at any given time. The number of tanks allocated to each cohort (Table 5.1) was determined through back calculation based on maximising the quantity of harvestable adult urchins given the total number tanks available (42), not exceeding a stocking density of 20% coverage (Chapter 2) and a mortality/culling rate of 35.58% over the culture cycle. This mortality rate is calculated to simplify the allocation of tanks per cohort, thereby streamlining the TAN emission module and management practices. This mortality rate is higher than has been observed in previous trials. However, *T. gratilla* individuals frequently show variations in growth. Certain individuals grow at a slow rate, which could reduce economic performance of the farm. Therefore, culling of these individuals may be necessary.

Table 5.1. Overview of the production cycle where weaned T. gratilla are first included into the system at 10 mm. Every month, as their test diameter increases, the cohort is moved into other tanks until they reach harvestable size.

Month in production cycle	**Minimum diameter (mm)**	**Number of tanks per cohort**
1	10	2.0
2	21	3.5
3	31	5.0
4	38	6.5
5	44	7.5
6	49	8.5
7	53	9.0

Urchin biomass in each tank was estimated by multiplying the number of individuals by the average individual mass predicted. To ensure simplicity, it was assumed that there was no inter-individual variability of growth between urchins within a cohort. Therefore, all urchins were assumed to be the same mass. This greatly reduces the complexity of the model and its computation time. Inter-individual variability is not necessary for the scope of this model (Chary *et al.*, 2022).

5.2.4.3 *T. gratilla* feed requirements

The quantity of feed required per feeding event was determined by multiplying the total urchin biomass by the recommended percentage of the specific feed. The feeding quantity and frequency are deduced from maximum *T. gratilla* values determined previously (Chapter 2), the literature (Cyrus *et al.*, 2015; Shpigel *et al.*, 2018; Shpigel and Erez, 2020), and experience from the pilot farm. The total amount of feed required for each cohort was calculated on a weekly basis to account for the growth of each cohort of urchins over the month. While the urchin biomass of the urchin growth model increases in a daily timestep, feed requirements would not be re-calculated daily on a farm. A weekly timestep was used because it is more practical for a commercial farm.

Three feeding scenarios are modelled. Each have their own input requirements calculated as follows:

A. *Ulva* only, where fresh *Ulva* is supplied throughout the production cycle at a rate of 6% of urchin mass daily;
B. Pellets only. The formulated pellets are supplied at 1.5% of urchin mass four times per week for the entire production cycle; and
C. *Ulva* and pellet combination. Urchins in the production cycle from zero to four months (somatic growth phase) are fed *Ulva* at 6% of their body mass daily, while urchins in the cycle from five to seven months (gonad enhancement phase) are fed pellets at 1.5% four times a week.

5.2.4.4 *T. gratilla* gonad production

The gonad production was estimated by multiplying the gonadosomatic index (GSI) by the total mass of the harvest urchin cohort. Two different average (± standard deviation) GSIs were assumed depending on the feed urchins were provided. For *Ulva* fed urchins (Scenario A), the GSI is lower than those fed pellet at least three months prior to harvest (Scenario B and C; Cyrus, 2015a, Chapter 2).

- Scenario A: GSI of 13.11 ±3.42% (Chapter 2).
- Scenarios B and C: GSI of 22.57 ±4.8% (Cyrus 2015a; de Vos, unpublished).

5.2.4.5 *Tripneustes gratilla* Total Ammonia Nitrogen (TAN) emission submodel

The production of TAN by fed organisms in aquaculture is frequently predicted using mechanistic models such as nitrogen retention or bioenergetic models (Chary *et al.*, 2022). These models rely on understanding, describing and formalising the underlying processes that influence the outcome. There are two reasons this approach is not used in this study. As *T. gratilla* is a new aquaculture candidate species there is data scarcity and thus the required parameters for these mechanistic models are largely not available. For the retention model specifically, two important parameters could not be determined. The digested nitrogen (nitrogen retained by the urchin for growth) and nitrogen retention apparent digestibility coefficient (ADC) for *Ulva* has not been clarified in the literature. Furthermore, these input variables could not be determined during the data collection period of this study for various reasons, including unavailability of juveniles. The second reason this family of models, and specifically nitrogen retention, is not utilized is due to difficulties of creating an accurate model. This is demonstrated by TAN production rates, based on nitrogen emission models, for the same species differing by a factor of 10 in the literature (Wheaton *et al.*, 1994). This is not surprising as there are numerous highly complex factors which have non-linear and interacting effects on TAN emission (Yu *et al.*, 2021, chapter 4). Therefore, to get accurate TAN emission predictions using a mechanistic approach would likely require separating, quantifying, understanding and accurately predicting the influence of each factor (such as microbial communities), as well as the interactions between all factors.

For these reasons, this study predicts TAN emissions through a relatively novel “black box” approach using an empirical regression model. While countless factors influence TAN, the designer and manager of the aquaculture facility only has control of relatively few factors (such as stocking density, flow rate, feed quantity, sludge removal etc). Therefore, these controllable factors are examined as dependent variables in a regression model with the independent variable being TAN emissions. A similar approach has been shown to accurately predict TAN concentration in *Ctenopharyngodon idellus* (grass carp) pond aquaculture (Yu *et al.*, 2021) and nitrogen emissions from cage aquaculture of various finfish species (Islam, 2005).

In this study, generalised, additive models (GAMs) were applied to describe and predict the TAN emission of *T. gratilla* culture systems. GAMs create a response variable which is dependent linearly on smoothing functions of the predictor variables (Hastie, 1992). The linearity of the model allows for easy interpretation, while the ability to regularize the predictor functions reduces the probability of overfitting (Wood, 2006).

5.2.4.5.1 Training data collection

To create the GAM model, data were collected from an experiment where 18 tanks were stocked with *T. gratilla* exposed to various feed types (fresh *Ulva* or pellets), feed quantity, exchange rates, stocking densities, size of urchins and number of urchins (Table 5.2). Water samples were taken from the tanks' inflows and outflows at specific time intervals post feeding. The TAN emissions were calculated by subtracting TAN concentration of each tank's outflow by the inflow. This is fully described in Chapter 4.

Table 5.2. A description of key variables in the training data. The sample size was 59.

	Min.	**Mean**	**Max.**
Stocking density (kg.m^{-3})	19.413	49.062	57.926
Exchange rate (turnover/h)	0.495	1.334	1.950
Total ammonia nitrogen emission (mg/l)	0	0.001	0.029

5.2.4.5.2 Variables of importance

An ensemble supervised learning method known as random forest analysis (Breiman, 2001) was applied to determine the extent of influence that the six explanatory variables (listed above) had on the TAN production in the training data. This method relies on the construction of multiple decision trees, which are algorithms created using random subsets of the explanatory data to predict the response. These "trees" are grouped into a "forest" where their arithmetic means are used for prediction. This process indicates the importance of each variable, thus allowing for variable selection when creating the GAM model.

5.2.4.5.3 GAM model construction

Model selection utilised various tools including Akaike's Information Criterion, deviance explained, and the importance of individual variables determined by both the random forest analysis and biological understanding (Chapter 4). Based on this, the model used the following predictor variables:

- Time from last being fed (hours), which was differentiated by feed types (pellets or *Ulva*);
- The quantity of feed supplied relative to tank volume, which is a function of urchin stocking density (kg.m^{-3}), as described in Section 2.5.3.; and
- Exchange rate of the urchin aquaculture system (turnovers per hour).

There were four knots, specific points where polynomial functions meet, for the time from feeding and three knots for stocking density and exchange rate. A shrinkage version of cubic regression splines ("cs") was found to be optimal. As the data set was relatively small (n= 59), strict regularization of smoothing was required to avoid overfitting. This involved only using a mixed model approach via restricted maximum likelihood (REML), which is also known to be highly efficient when avoiding undersmoothing (Wood, 2011). Post-hoc inspections further ensured that overfitting was avoided. Various functionality checks were conducted for this model. For all predictors, there was no indication of concurvity (concurvity values < 0.7), and the basis dimension (k) indicated knots were sufficient ($p >$ 0.3). See Appendix I for various internal model/assumption validation plots for the GAM model predicting TAN.

5.2.4.5.4 Determination of total TAN emission from urchin system

The GAM model was used to predict the TAN emissions for one tank from each urchin cohort on an hourly basis, over the monthly stocking cycle for each feeding scenario at the given feed quantity relative to tank volume, flow rate and feed type. It was necessary to calculate this at an hourly timestep to determine if the TAN concentration would exceed toxic levels for *T. gratilla* (objective 2) as the time from feeding had a strong influence on TAN emission (Chapter 4).

The hourly predicted TAN emission from a tank of each cohort was multiplied by the number of tanks allocated to each cohort (Table 5.1). These values for each cohort were added together and divided by the total number of tanks within the system (42). This provided the TAN emission of the effluent coming from the urchin production system into the *Ulva* raceway.

The daily mass of nitrogen derived from the TAN emission of the urchin system was also calculated by finding the average daily TAN concentration in the effluent (on a weekly basis), then multiplying this number by the flow rate (178 500 $l.h^{-1}$). This mass value was multiplied by the proportion of nitrogen mass present in TAN.

5.2.4.6 External validation of *T. gratilla* submodels

These submodels have not been used in farm-scale modelling prior to this study. Therefore, to increase confidence in their predictions, validation using externally sourced data (i.e., data not used to create the submodels) was conducted.

5.2.4.6.1 Individual *T. gratilla* growth and diameter to mass relationship submodels: external data sources

The individual growth model and the size to mass relationship were validated using two different data sets. Data from Cyrus et al (2015a) was used to validate the individual growth model. This dataset included observed size (n = 282) from urchins with an approximate test diameter of 30 to 80 mm over 32 weeks, while being fed 20U pellets, fresh *Ulva* or a combination of the two diets. A dataset containing 2 987 paired urchin mass and test diameter measurements collected over the various urchin trials discussed in Chapter 2 was used to validate the size to mass relationship.

5.2.4.6.2 Description of external validation data for TAN emission model

This experiment followed the same structure and concept as that described in Section 2.5.5.1, and the trial was conducted at the same facility on the 22/08/2022. Essentially, TAN emission was determined from various tanks containing *T. gratilla* at specific time points following feeding, while exposed to different stocking densities, flow rates, fed different feed types (*Ulva*, pellets or no feed), and particularly tank sizes (Table 5.3). The focus of this experiment was to assess if the TAN predictions provided by the GAM model would scale adequately. The training data was collected from small 0.04 m^3 tanks. The external validation data was derived from mostly larger tanks (maximum of 1.386 m^3). If the model accurately predicts the TAN emissions from these larger systems, it provides confidence in its farm-scale predictions. Unfortunately, a power outage occurred overnight and most of the TAN measurements for the *Ulva* fed treatments were lost. In total, there were 15 observed TAN emission values, four from *Ulva*-fed tanks and 11 from pellet-fed tanks.

Table 5.3. A baseline description of some variables of the external validation data.

	Min.	**Mean**	**Max.**
Stocking density (kg.m^{-3})	5.4473	31.602	41.250
Exchange rate (turnovers.h^{-1})	0.338	0.815	1.044
Tank volume (m^{-3})	0.04	0.640	1.386
Total ammonia nitrogen emission (mg/l)	0.001	0.007	0.016

5.2.4.7 Model validation criteria

The mean absolute percentage error (MAPE, Equation 5.3) was calculated for the urchin growth and diameter to mass sub-models. This provides an easily interpretable error value as percentage, which is not easily skewed by outliers.

$$\text{MAPE} = \frac{1}{\text{n}} \Sigma \left(\frac{\text{Pi} - \text{Oi}}{\text{Oi}} \right) \tag{5.3}$$

Where:

P_i = predicted value for the ith observation in the dataset

O_i = observed value for the ith observation in the dataset

n = sample size

The TAN emission model, however, was reported as root-mean-square error (RMSE, Equation 5.4). While not as interpretable and more likely influenced by outliers, it is a more appropriate metric for this model. This is because MAPE can suffer from a division by 0 error, unlike RMSE. Also, many of the TAN emission values are close to zero (Botchkarev, 2019).

$$\text{RMSE} = \sqrt{\frac{\Sigma(\text{Pi} - \text{Oi})^2}{\text{n}}} \tag{5.4}$$

Additionally, a regression method of model validation was applied to all the urchin submodels. The observed and predicted results were plotted against each other, and a straight line of best fit was created via ordinary least squares (OLS). A good model, where the predicted and observed values are similar, will result in the line of best fit having:

- A slope very near 1 (as the observed and predicted values would be directly proportional);
- the intercept would be near 0, as there would be no bias; and
- the coefficient of determination (R^2) value would be near 1, due to high correlation.

The coefficients and their associated standard errors are applied in a T-test or Wald-test to determine whether they differ significantly from their expected values (Jusup *et al.*, 2009). Based on this, the performance of models can be classified into one of the categories listed below in Table 5.4 (Portilla and Tett, 2007).

Table 5.4. The method of categorising the urchin submodels performances based on the regression between the predicted and observed values and various statistical tests of its coefficients and standard errors. Significantly is abbreviated to “sig.”.

Model category	**R^2 is sig. different from zero**	**Intercept is not sig. different to 0**	**Slope gradient is not sig. different to 1**
Poor	False	False	False
Fair	True	False	False
Good	True	One of the above is true	
Very good	True	True	True

5.2.5 *Ulva* production and TAN assimilation

The *Ulva* growth and nitrogen assimilation model described here is largely based on the model described by Solidoro *et al.* (1997). The specific growth rate of *Ulva* (μ_{growth}) is predicted using a basic multiplicative model (Equation 5.5), where the maximum observed specific growth rate (μ_{max}) is multiplied by the factor that is most limiting (Lehahn *et al.*, 2016; Martins and Marques, 2002; Solidoro *et al.*, 1997; Zollmann *et al.*, 2021). These limiting factors could be internal concentration of nutrients ($\mathrm{g(N)}$), light ($\mathrm{g(I)}$), temperature and/or salinity. This model assumes temperature and salinity to not be restrictive (Section 5.2.6). The value used for the highly sensitive observed maximum specific growth rate (μ_{max}) was 0.416 day^{-1} as this value, determined by Oca *et al.*, (2019), had high agreement with other studies (Bendoricchio *et al.*, 1994; de Guimaraens *et al.*, 2005; Duke *et al.*, 1989; Hadley *et al.*, 2015; Menesguen and Salomon, 1988; Parker, 1981).

$$\mu_{\text{growth}} = \mu_{\text{max}} * \min\{g(\text{I}) * g(\text{N})\} \tag{5.5}$$

To estimate the net growth (μ_{net}), it was necessary to subtract the natural loss of biomass over time through mortality and fragmentation. (Equation 5.6). This has been found to have a specific rate of 0.066 day^{-1} (Oca *et al.*, 2019).

$$\mu_{\text{net}} = \mu_{\text{growth}} - \lambda \tag{5.6}$$

To determine the maximum potential production of the *Ulva* raceway, given the model assumptions (Section 5.2.6), the light limited production factor was first determined ($g(\text{I})$). This was determined using Equation 5.7. The half-light saturation constant (K_I) was concluded to be 20 µmol photons m^{-2} s $^{-1}$ based on calibration from scale appropriate experimental data (Chemodanov *et al.*, 2019; Zollmann *et al.*, 2021). Photosynthetically active radiation (PAR) is the ratio of the sunlight that is suitable for photosynthesis and is widely agreed to be 0.43 (Mõttus *et al.*, 2013).

$$g(\text{I}) = \frac{\text{I}_{\text{average}}}{\text{K}_\text{I} + \text{I}_{\text{average}}} * \text{PAR} \tag{5.7}$$

Average photon irradiance in the water ($\text{I}_{\text{average}}$), which is the average amount of light the *Ulva* is exposed to in the raceway, is determined via Equation 5.8. This uses the incident photon irradiance at the water surface (I_0), which is established in Section 5.2.6. This value is reduced within the tank by two factors:

- The depth of the raceway (Z) multiplied by the water light extinction coefficient (K_0), which has been found to be 1.5 m^{-1} in an aquacultural context (Oca *et al.*, 2019); and
- The stocking density (SD) of the *Ulva* multiplied by the *Ulva* light extinction coefficient (K_a), which was similarly found to be 0.01 m^2 gDW^{-1} (Oca *et al.*, 2019).

$$\text{I}_{\text{average}} = \frac{\text{I}_0}{\text{K}_0\text{Z} + \text{K}_\text{a}\text{SD}} \left[1 - \exp\left(-(\text{K}_0\text{Z} + \text{K}_\text{a}\text{SD})\right)\right] \tag{5.8}$$

Once the light limited *Ulva* production was calculated, the nutrient limitation $g(\text{N})$ on growth was calculated using a flexible adaptation of Droop's cell quota model (Solidoro *et al.*, 1997). It was assumed the only limiting nutrient was nitrogen supplied by TAN emitted from the *T. gratilla* effluent (Section 5.2.6). This limitation was determined by the internal nitrogen content of the *Ulva* (N_{int}). The minimum nitrogen content (N_{min}) reported to be 10 mg N.g^{-1} DW (Cohen and Neori, 1991; Sfriso *et al.*, 1987). A nitrogen composition for *Ulva* was selected as the initial value (N_0). In our case, the "critical" value (N_{crit}) was chosen, 20 mg N.g^{-1} DW (Fujita, 1985). While this was considered a critical value because above this level *Ulva* is not nitrogen limited (Hanisak, 1983). The choice of value was relatively arbitrary as it would only influence the time taken to achieve a steady state. The growth constant $\mathrm{k_c}$ was set at 8 (Solidoro *et al.*, 1997).

$$\mathrm{g(N)} = \frac{\mathrm{N_{int} - N_{min}}}{\mathrm{N_{int} - k_c}} \tag{5.9}$$

The specific TAN uptake rate by *Ulva* ($\mathrm{V_{TAN}}$) was also calculated by a similar multiplicative model (Equation 5.10). This equation used the maximum TAN uptake rate determined as 5.2 mg N g DW^{-1} h^{-1} (Solidoro *et al.*, 1997), $\mathrm{f_1(N)}$ (Equation 5.11) and a factor ($\mathrm{f_2([TAN])}$) which limited TAN uptake based on the concentration of TAN in water (Equation 5.12).

$$\mathrm{V_{TAN}} = \mathrm{V_{max}} * \mathrm{f_1(N)} * \mathrm{f_2(TAN)} \tag{5.10}$$

The determination of $\mathrm{f_1(N)}$ required the same inputs as $\mathrm{g(N)}$ and the maximum nitrogen content observed in *Ulva* ($\mathrm{N_{max}}$) reported to be 45 mg N g^{-1} DW (Cohen and Neori, 1991; Sfriso *et al.*, 1987).

$$\mathrm{f_1(N)} = \frac{\mathrm{N_{max} - N_{int}}}{\mathrm{N_{max} - N_{min}}} \tag{5.11}$$

This TAN concentration factor ($\mathrm{f_2([TAN])}$) was calculated by the expected TAN concentration of the water body (Equation 5.12). This value for this model was estimated from the daily average TAN emission from the *T. gratilla* system. A half saturation constant for TAN ($\mathrm{K_{TAN}}$, 0.7 mg.l^{-1}), is based on findings from (Fujita, 1985) due to the experiment being most appropriate as reported by Solidoro *et al.* (1997).

$$\mathrm{f_2([TAN])} = \frac{\mathrm{[TAN]}}{\mathrm{[TAN] + K_{TAN}}} \tag{5.12}$$

To determine the change of *Ulva*'s nitrogen composition, and therefore be able to calculate its growth and nitrogen uptake, Equation 5.13 was applied. This indicated the presence of a steady state of $\mathrm{N_{int}}$.

$$\frac{\mathrm{dN_{int}}}{\mathrm{dt}} = \mathrm{V_{TAN}([TAN], N_{int})} - \mu_{\mathrm{growth}} * \mathrm{N_{int}} \tag{5.13}$$

5.2.6 Model assumptions

Air was supplied via bubbling via perforations in the floors of the tanks. Oxygen has not been observed to be a significant limitation of *T. gratilla* culture due to their low levels of oxygen consumption (Mos *et al.*, 2012). Based on these studies and observations from the pilot urchin-*Ulva* IMTA system at Buffeljags Abalone farm, it was assumed oxygen would not be a limiting factor of production. Furthermore, due to the water movement driven by the aeration, the water quality within each tank was assumed to be homogenous.

The water temperature at the farm was assumed to be a constant 25°C. While this may not be entirely realistic, it greatly simplified the model and was a reasonable assumption. This is because it is the temperature *T. gratilla* is generally cultured in (Dworjanyn *et al.*, 2007). Therefore, coastal regions that have water of this temperature would be most suitable for their production because heating water would be an economical and environmental disadvantage. Regions with 25°C water are generally equatorial, where there is little variation in climate (temperature) throughout the year (Jeffs, 2013; Locarnini *et al.*, 2018). Similarly, salinity is assumed a constant 35ppm.

Temperature and salinity are assumed not to be limiting factors for *Ulva* production or nitrogen uptake. While there are certain species of *Ulva* that would be limited in this conceptual system (Martins *et al.*, 1999), other *Ulva* species/strains have been shown to achieve near maximum growth rates in these conditions (Bews *et al.*, 2021; Xiao *et al.*, 2016). It is assumed the *Ulva* selected for this system will be appropriate for its conditions, thus not limited by temperature or salinity.

The average incident photon irradiance (E_0) was assumed to be a constant 2 500 µmol photons m^{-2} s^{-1} throughout the year with a 12/12 hour daily light cycle. This static value was chosen to increase simplicity of the model and because it is realistic due to the relatively stable climate expected in *T. gratilla* production regions, as previously discussed. This value was reported as a mean for the tropics (Lüning, 1991) and is near that reported from regions suitable for *T. gratilla* production due to appropriate water temperatures (Rosenberg *et al.*, 2011; Zollmann *et al.*, 2021). The stocking density of 1 $kg.m^{-2}$ of *Ulva* was chosen as it is considered optimal for both nitrogen content and production (Neori *et al.*, 1991). The urchin system itself was assumed to be shaded for reasons discussed in Chapter 4.

The ambient TAN concentration of the incoming seawater will have a strong influence on the performance of this system. Seawater with TAN levels over 1 mg/l has been reported

(Wright *et al.*, 2019) due to anthropogenic causes, which would probably result in mass mortality of urchins. However, this study assumes most of its production will occur in the warm oligotrophic waters suited to *T. gratilla* production. It has been found that these waters generally rarely exceed TAN concentrations of 0.001 mg/l (Rees *et al.*, 1999). As this is already a nearly insignificant value and it would be further diluted due to recirculation, this model assumes that the TAN (and other dissolved nitrogen species) derived from the incoming seawater is zero. Therefore, all TAN in this system is derived from the urchins and their feed.

While the urchin system will produce nitrate and phosphate (Chapter 4), this model assumes this will not influence the production of either the urchins or the *Ulva*. The reasons for these nutrients not influencing urchin production are discussed in Chapter 4. It is assumed that phosphate will not limit growth or nitrogen assimilation of *Ulva* as the internal critical phosphorus content of *Ulva* is considerably lower than nitrogen (Björnsäter and Wheeler, 1990) and is very near the minimum quota value (Lavery and McComb, 1991). Furthermore, the ratio of phosphate to nitrogen in aquaculture effluent is generally very high, thus highly unlikely to be a limiting factor for *Ulva* production (Hadley *et al.*, 2015). Nitrate can be a nitrogen source, which will support growth of *Ulva*. However, nitrate uptake by *Ulva* is effectively inhibited in the presence of ammonia (Ale *et al.*, 2011; Neori *et al.*, 1996; Thomas and Harrison, 1987). As the effluent from the urchin system is constantly being added into the *Ulva* raceway, it seems likely there will always be some presence of ammonia within the system. Therefore, the nitrate is unlikely to contribute meaningfully to *Ulva* growth, this assumption is confirmed in the discussion.

5.3 Results

5.3.1 Model calibration

5.3.1.1 Generalised additive model (GAM) calibration

The random forest analysis indicates that among the variables tested, exchange rate had the strongest impact (16.15% importance) on the TAN emission from *T. gratilla* aquaculture systems (Fig. 5.4). This was followed closely by time from being fed (15.2%). The type of feed; fresh *Ulva* or artificial feed, also had a great influence on the output of TAN. The quantity of feed, which is dependent on stocking density, had a stronger influence than the number of individuals in a tank. It is also a more scalable and descriptive metric as stocking density is represented by the mass of urchins over the volume of water. For these reasons, the number of individuals was dropped as an explanatory variable in the predictive models. The average individual mass of the urchins was also not applied in the predictive models as it did not have a strong influence on the response variable.

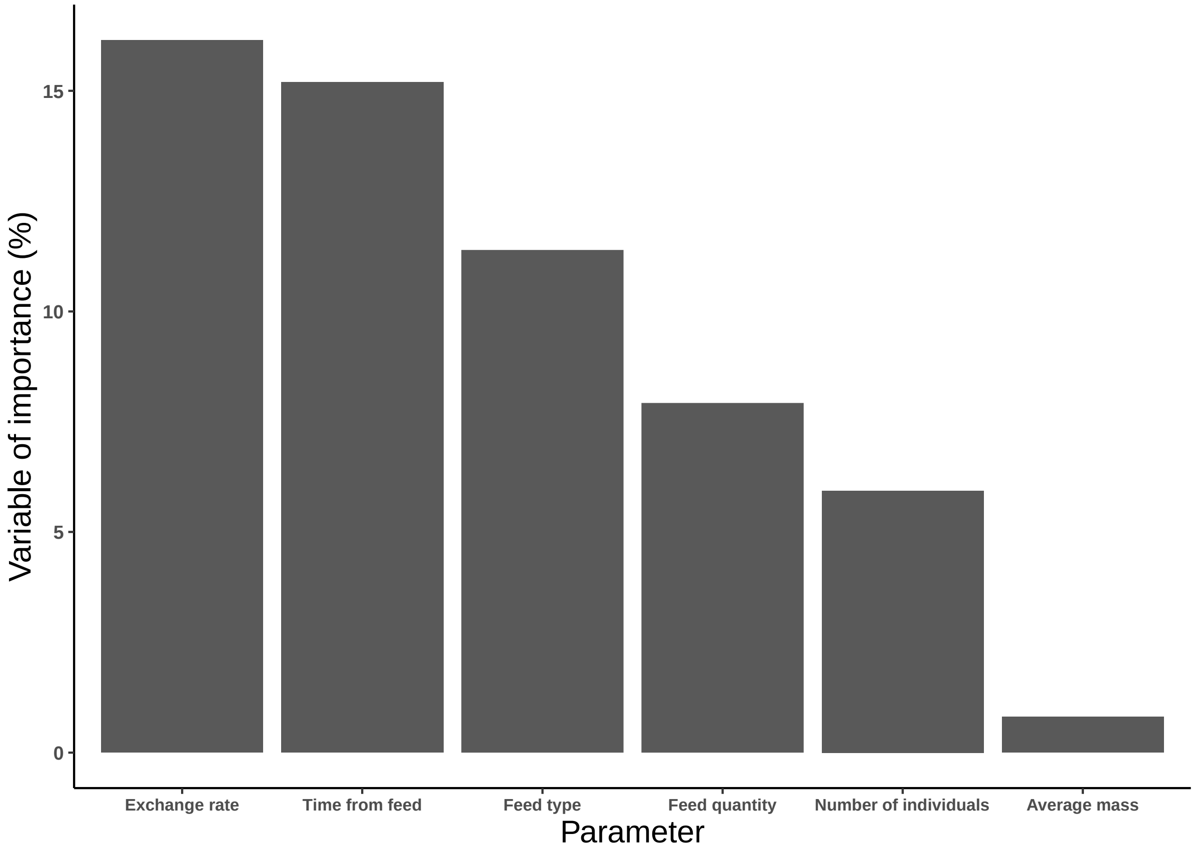


Fig. 5.4. Importance of specific variables in predicting the emission of total ammonia nitrogen from *Tripneustes gratilla* systems based on a random forest analysis

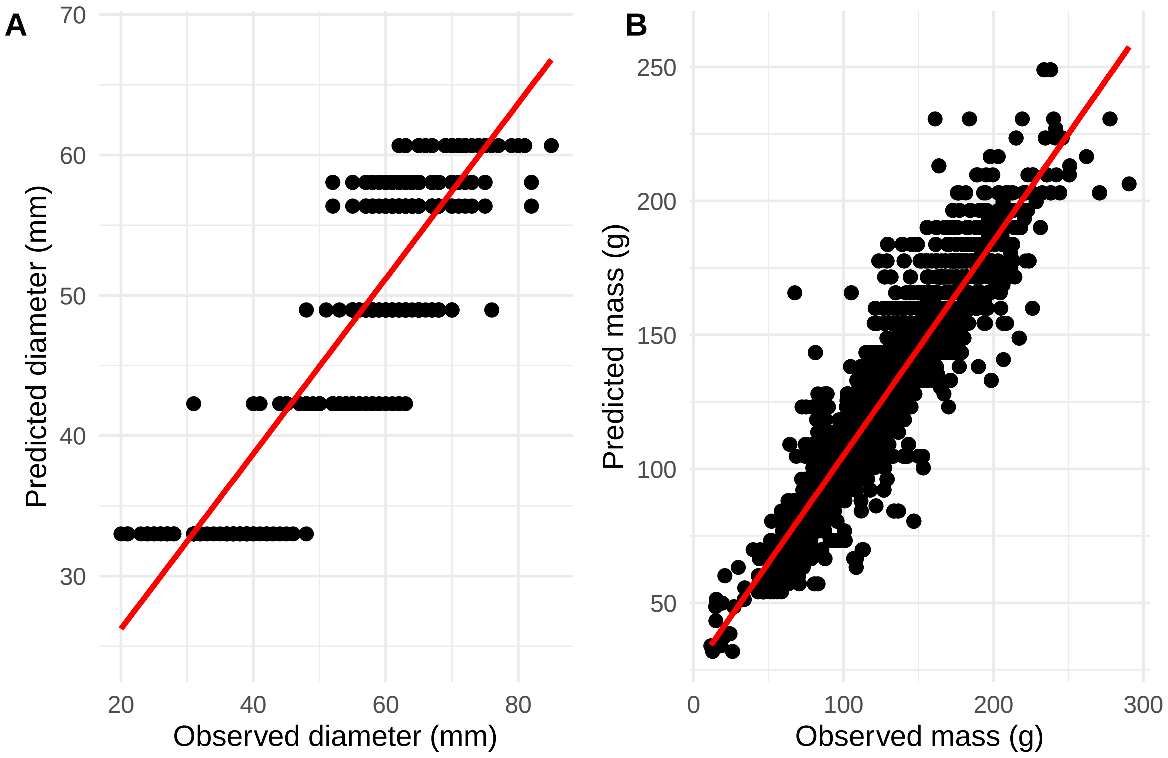
5.3.2 Validation of models

5.3.2.1 Validation of growth and diameter to mass sub-models

The predictions derived from the Johnson's differential growth equation (Dafni, 1992) were found to be on average 13.61% lower (i.e., 7.71 mm) than the observed values of *T. gratilla* growth from an external data source (n = 281), indicating underestimation of the predicted size. The coefficient of the slope (0.62, Table 5.5) indicates this model's underestimation is predominately at the lower end of the range. This coefficient and its standard error were found to be significantly different to 1. The intercept was significantly different to 0 and the

R^2 value was found to be significantly different to zero (Table 5.1). Therefore, this model would be categorised as “fair”. The mean absolute percentage error (MAPE) was 18.31%.

The predicted values for the diameter to mass conversion model underpredicted the mass by on average 0.67% (i.e., 0.87 g) when compared to the external data set. The MAPE was 0.1%. It should be noted that this data set was considerably larger (n = 2987). When the coefficients of the OLS fitted line were tested, it was similarly found that the slope, intercept and R^2 coefficients differ significantly from their null hypotheses and therefore this model is also classified as “fair” (Table 5.5). This concludes that the urchin production model therefore generally underestimates growth.



Figs. 5.5 (A-B). The predicted and observed values of test diameter (A) and diameter to mass (B) submodels. The red line indicates a straight line constructed by ordinary least squares (OLS) regression with coefficients reported in Table 5.5. The OLS line of Fig 5.5.A had a slope of 0.624, intercept of 13.743 and R^2 value of 0.762. The six horizontal clusters in Fig. 5.5.A reflect the urchins being measured on six different occasions. Therefore, the model will predict the urchins to be the same mass. The OLS line of Fig 5.5.A had a slope of 0.8, intercept of 24.986 and R^2 value of 0.85.

Table 5.5. The coefficients, standard deviations, associated tests and given category of ordinary least squares (OLS) regression between the observed and predicted data of the individual urchin growth submodel and of the size-mass relationship.

MODEL	COEFFICIENT	OLS ESTIMATE	STANDARD ERROR	NULL HYPOTHESIS	TEST STATISTIC	P-VALUE	MODEL CATEGORY
GROWTH	Slope (k)	0.624	0.021	k = 1	t = -18.062	<0.001	"Fair"
	Intercept (I)	13.743	1.210	I = 0	t = 11.333	<0.001	
	Determination (R^2)	0.762	-	$R^2 = 0$	$F_{(1, 280)} = 892.04$	<0.001	
DIAMETER TO MASS	Slope (k)	0.800	0.006	k = 1	t = -32.468	<0.001	"Fair"
	Intercept (I)	24.986	0.833	I = 0	t = 29.995	<0.001	
	Determination (R^2)	0.850	-	$R^2 = 0$	$F_{(1, 2986)} = 16882.84$	<0.001	

5.3.2.2 Validation of *T. gratilla* TAN emission model

A comparison between observed and predicted values from an external data set provided evidence that the GAM model can provide accurate predictions of TAN emissions from *T. gratilla* aquaculture systems of various sizes. The root-mean-standard error (RMSE) was low (0.006). Regression between predicted and observed values indicated high correlation between these values ($R^2 = 0.713$) however there is an apparent logarithmic response (Fig. 5.6). However, the corresponding tests for the coefficients and standard errors of the line of best fit resulted in it being categorised as a "very good" model (Table 5.6).

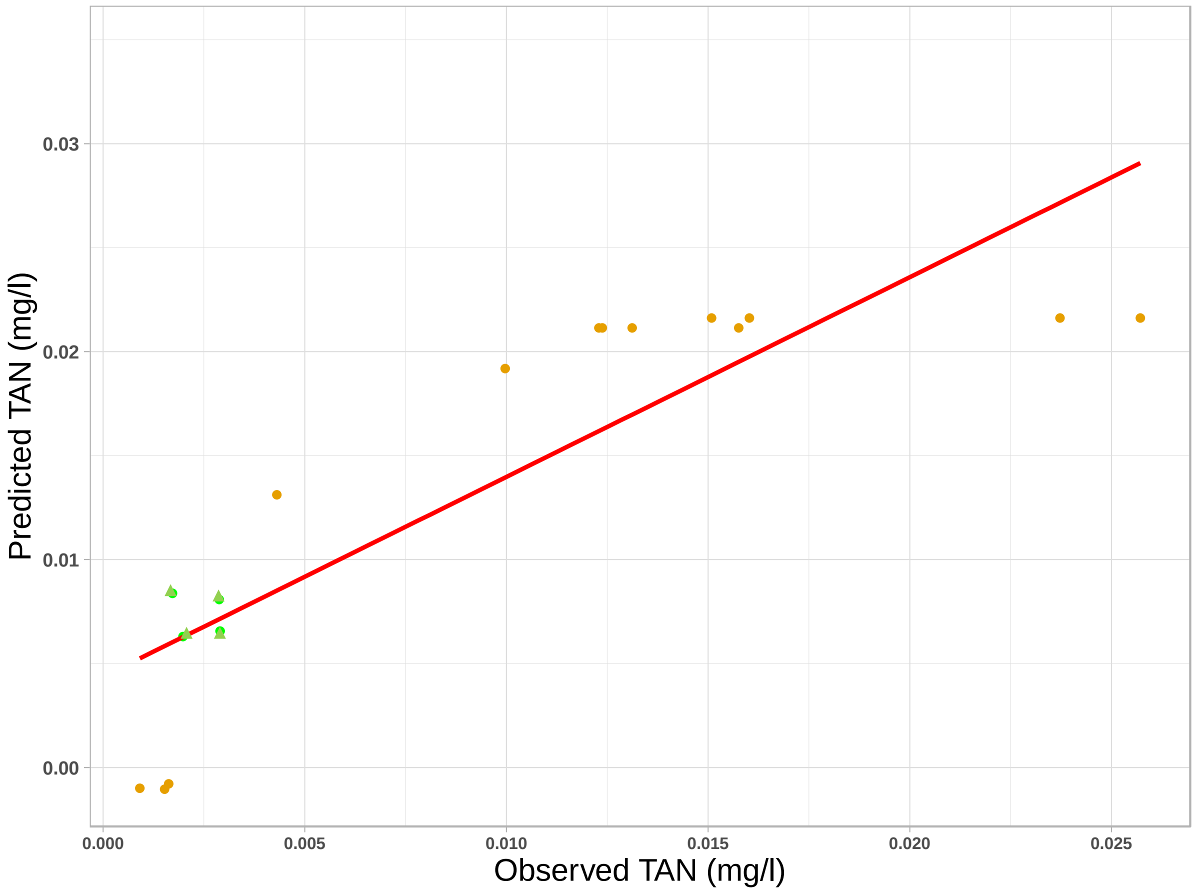


Fig. 5.6 The predicted and observed values for total ammonia nitrogen (TAN) concentration for various *Tripneustes gratilla* TAN emission models. The red line indicates a straight line constructed by OLS regression with coefficients reported in Table 5.6. The green triangles represent the TAN emission of urchin systems where *Ulva* was supplied as feed, while the orange dots are for pellet-fed urchins.

Table 5.6. The coefficients, standard deviations, associated tests and given category of ordinary least squares (OLS) regression between the observed and predicted data of the various *T. gratilla* TAN emission models.

MODEL	COEFFICIENT	OLS ESTIMATE	STANDARD ERROR	NULL HYPOTHESIS	TEST STATISTIC	P-VALUE	MODEL CATEGORY
GAM	Slope (k)	0.961	0.151	k = 1	t = -0.258	0.799	'Very good'
	Intercept (I)	0.004	0.002	I = 0	t = 2	0.06	
	Determination (R^2)	0.713	-	$R^2 = 0$	$F_{(1,16)} = 37.246$	<0.001	

5.3.3 Model use

5.3.3.1 *T. gratilla* biomass accumulation over production cycle

At the beginning of each month, there will be nearly 9500 kg of urchins in the system (42 tanks). By the end of each month, these 363 511 urchins will have increased to a total biomass of 13200 kg (Fig. 5.7). At this point, every cohort will need to be stocked down to retain the stocking density below 20% coverage, aside from cohort seven (that have been in the production cycle for seven months), which will be harvested. The minimum volumetric stocking density, 2.9 kg.m^{-3}, is observed on day zero of cohort one. This density gradually increases to reach a maximum on day 30 in cohort seven, which will have a density of 42 kg.m^{-3}.

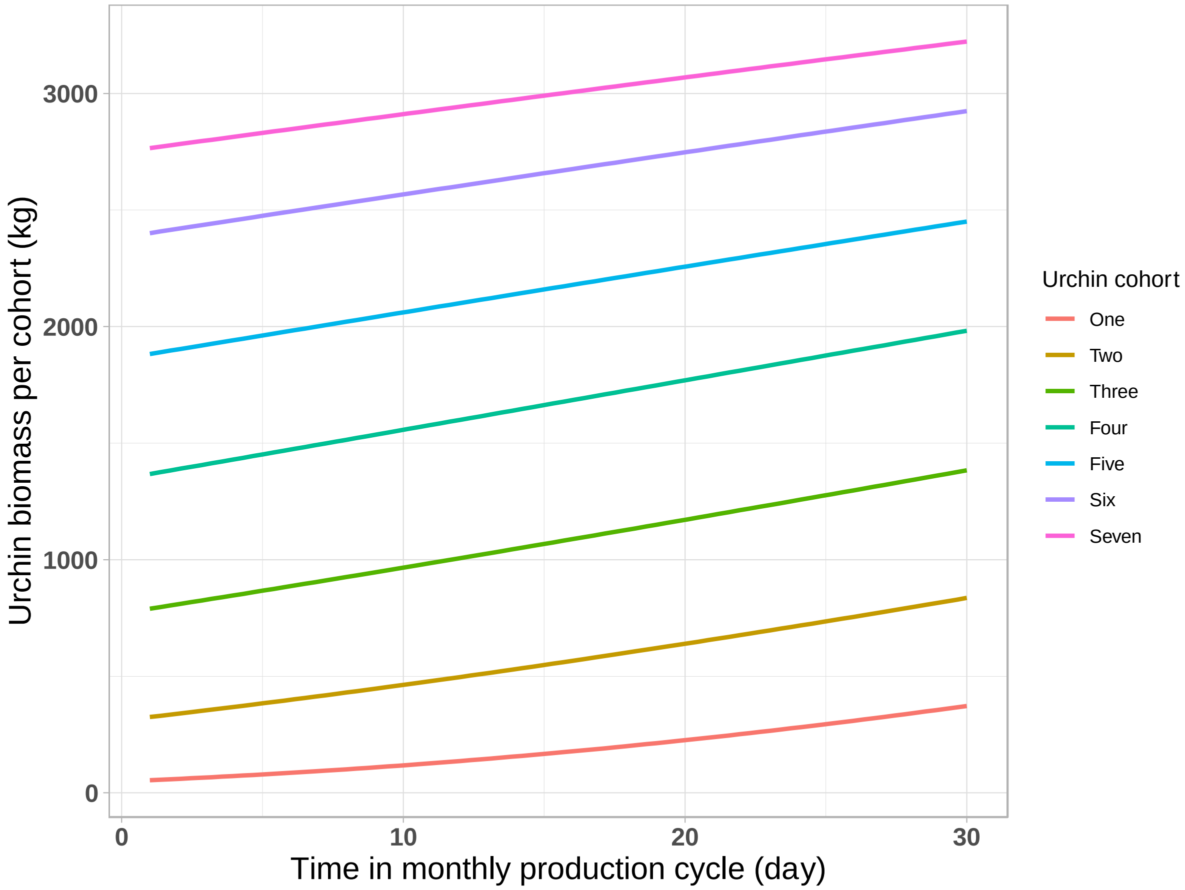


Fig. 5.7. The increase in total urchin biomass within each cohort over the monthly production cycle. Each colour represents a different cohort, starting with cohort one being the juvenile (*ca.* 10 mm) urchins, which have been in the production cycle for a month or less. It ends with the cohorts that have been in the system for seven months (cohort seven), which will be harvested at the end of the cycle.

5.3.3.2 *Tripneustes gratilla* production system TAN emission

For Scenario A, where fresh *Ulva* is fed to all urchin cohorts, the average TAN concentration from the conceptual urchin system (42 tanks each with a volume of 8.5 m^3) into the *Ulva* raceway (150 m^3) is 0.012 mg/l. The minimum TAN concentration is 0.008 mg/l, observed an hour prior to each feeding in the first week of the production cycle. The maximum TAN concentration is 0.017 mg/l, observed 10 hours after feed is provided in the last week of the production cycle. The daily average nitrogen production from this urchin system, derived from TAN, is 41.508 g. The greatest TAN concentration within a single tank for this scenario was 0.018 mg/l. This was observed in the tanks of cohort seven, 10 hours after feeding and in the last week of the production cycle.

The TAN concentrations show daily fluctuations dependent on feeding, where TAN concentrations are lowest just prior to feeding, rapidly increasing to a maximum at 10 hours after feeding (Fig. 5.8). The daily TAN concentrations are the same within each week but gradually increase week by week throughout the production cycle. This reflects the increase in feed quantity, which occurs once a week. This entire cycle will be identical every month, assuming the described monthly production cycle (Section 5.2.2) is applied. These patterns apply to all the feeding scenarios (Figs. 5.8, 5.9 and 5.10).

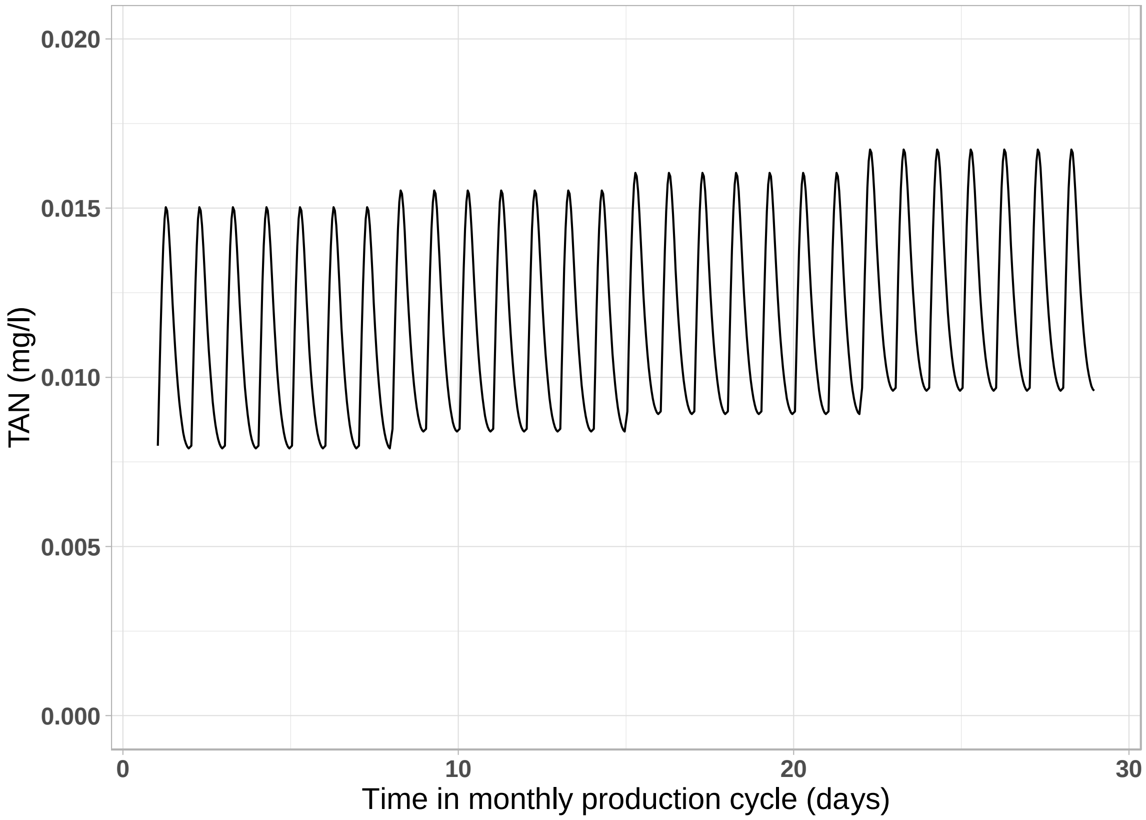


Fig. 5.8. The combined total ammonia nitrogen (TAN) concentrations from the all-urchin tanks into the *Ulva* raceway, where urchins are fed *Ulva* (Scenario A) over a 28-day production cycle. Note: This is the TAN production from the urchins and not including the ambient TAN concentration of the inflowing water, which this model assumes to be zero.

When pellets are provided to all urchin cohorts (Scenario B), the TAN concentration from the entire urchin system was on average 0.006 mg/l. While this average value is considerably lower than that of Scenario A, the fluctuations in TAN concentration were considerably higher with total system maximum of 0.023 mg/l and minimum of -0.005 mg/l. This negative emission value is discussed in Chapter 4. The maximum value was observed an hour after feeding and is depicted as a spike on top of the crest (Fig. 5.9). A maximum TAN concentration within a single tank for this scenario was 0.027 mg/l, from cohort seven, one hour after feeding and in the last week of the production cycle. The average daily TAN derived nitrogen production of the urchin system effluent when fed pellets was 20.708 g per day.

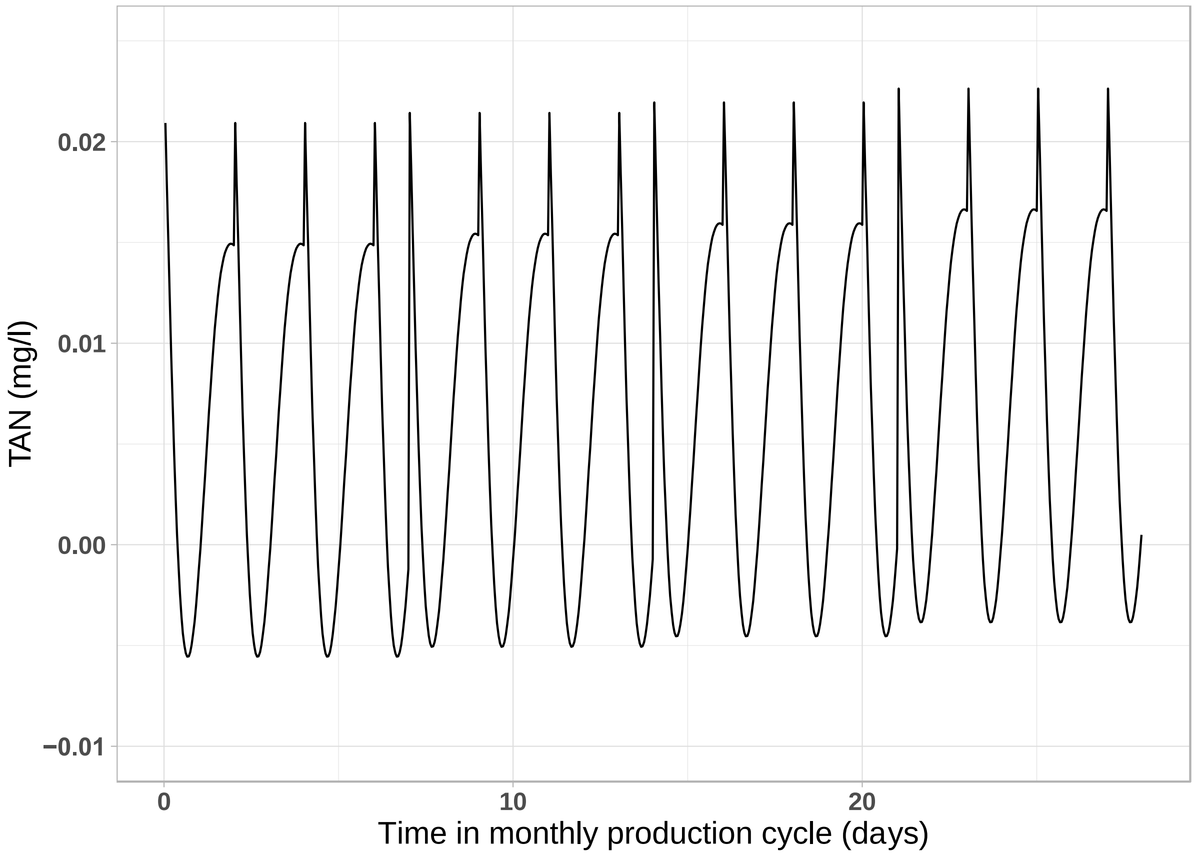


Fig. 5.9. The combined total ammonia nitrogen (TAN) concentrations/emission from the all-urchin tanks into the *Ulva* raceway where urchins are fed pellets (Scenario B) over the 28-day production cycle. The spikes in TAN represent the leaching as pellets are added into the system. The sharper spikes on days 7, 14 and 21 represent the change of weekly feeding regimes and the need to feed two days in a row, instead of every second day.

The TAN concentration of Scenario C, where a combination of fresh *Ulva* and pellets are fed, shows a similar pattern to that of Scenario B. Mostly notably, TAN spikes one hour after the pellet feeding (Fig. 5.10). However, there is evidence of the *Ulva*-fed tanks reducing some of the effects from the pellet-fed tank effluent, where these spikes reach a maximum of 0.017 mg/l and are therefore not as substantial as those in Scenario B. Similarly, the minimum point is not as extreme, at 0.001 mg/l. The mean was effectively the same as Scenario B at 0.006 mg/l. The average daily nitrogen production from TAN is 22.844 g per day. The maximum TAN concentration within a single tank for this scenario (scenario C) was the same as Scenario B.

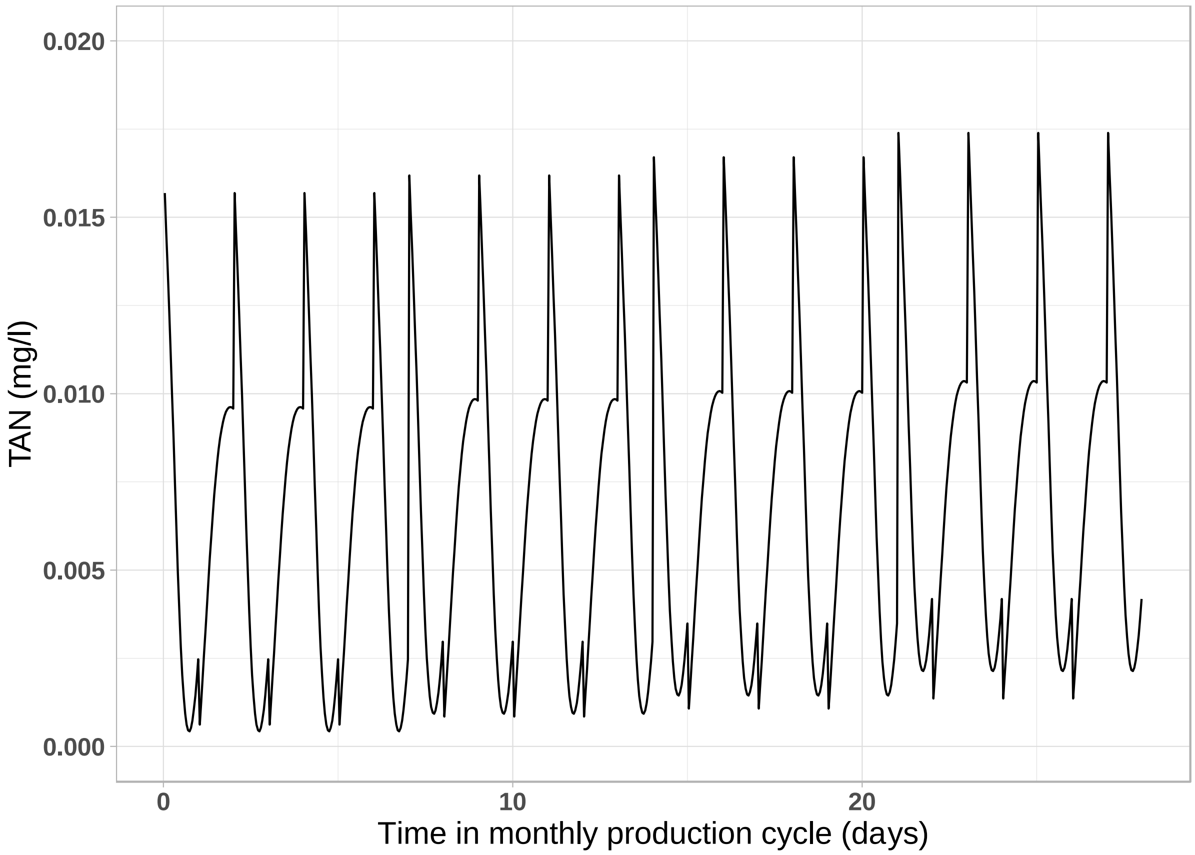


Fig. 5.10. The combined total ammonia nitrogen (TAN) concentrations from the all-urchin tanks (and all cohorts) into the *Ulva* raceway in feeding Scenario C, when fresh *Ulva* is provided to urchins in cohorts one to four and remaining cohorts; five six and seven, are fed pellets only to increase the size of the gonads.

5.3.3.3 *T. gratilla* gonad production

At the end of each month there are 3.22 t of harvestable whole urchin regardless of feeding scenario. Therefore, the annual whole urchin harvest will be 38.64 t. While total mass of urchins is unaffected, the quantity of gonad differs depending on the feed supplied in the final three months. The GSI is expected to be 22.57 ±4.8% for those fed pellets as a finishing diet (Scenario B or Scenario C) and 13.11 ±3.42% for urchin fed fresh *Ulva* only (Scenario A). Therefore, the gonad yield for the pellet fed urchins is likely to be between 0.57 and 0.88 t per month (Fig. 5.11). For *T. gratilla* fed *Ulva* for their entire life cycle, it would be between 0.31 and 0.52 t per month.

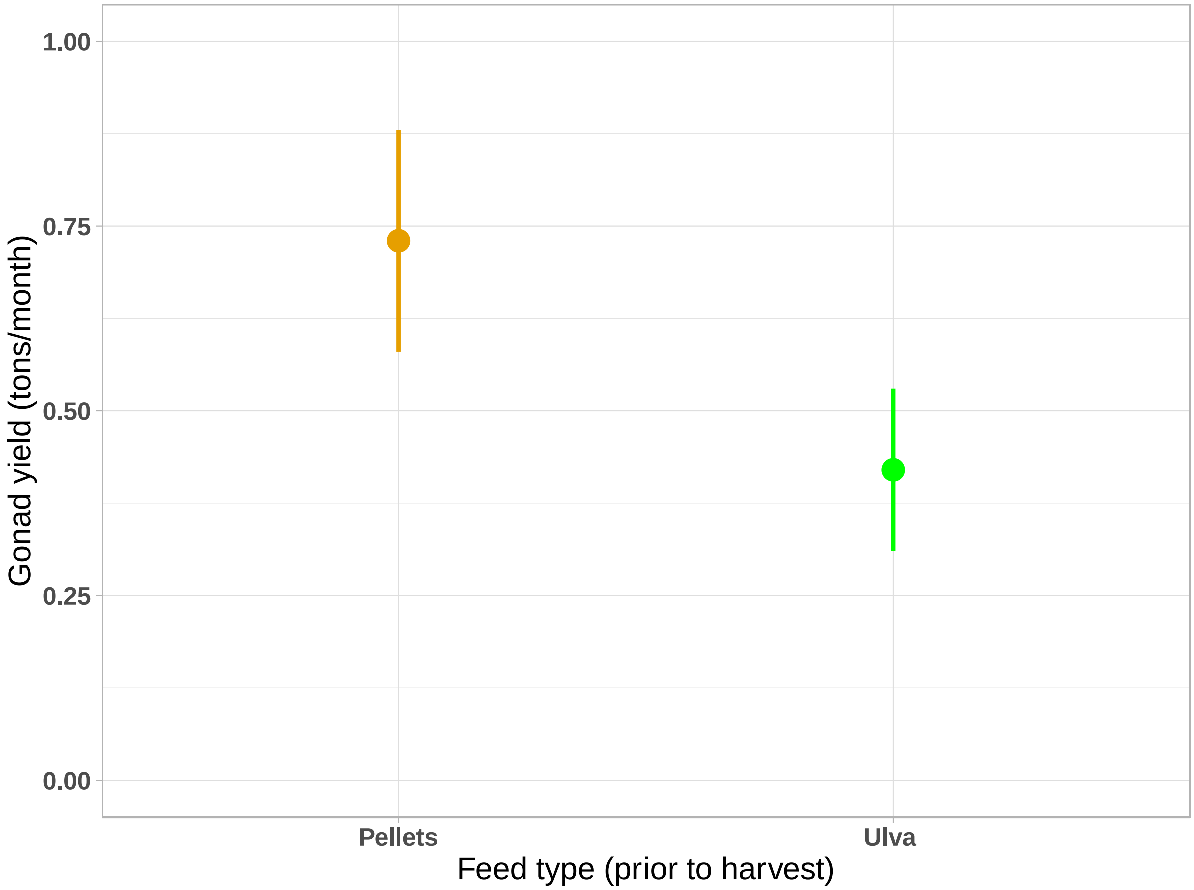


Fig. 5.11. The predicted monthly gonad yield (in metric tons) from the conceptual farm based on what is fed prior to harvest. Feeding Scenario B and C both have pellets (indicated by the brown line) fed prior to harvest, therefore, the same gonad yield is assumed. The *Ulva*-fed Scenario A gonad yield is indicated via the green line. The dot indicates the average expected production, and the lines suggest the standard deviation.

5.3.3.5 *T. gratilla* monthly feed requirements

When all urchins are fed fresh *Ulva* at a rate of 6% of their body mass daily (Scenario A), they will require 2.43 t of dried *Ulva* monthly (Table 5.7). When all urchins are fed pellets at 1.5% of their body mass four times a week (Scenario B), 2.57 t of these pellets will be required. These pellets are composed of 20% dried *Ulva*. As such, the formulation of these pellets will require 0.51 t of dried *Ulva*. Scenario C, where *Ulva* is fed for the first four months and then pellets are fed for the last three months, will require 1.33 t of pellets and 0.98 t of dried *Ulva*, which is a combination of the fresh *Ulva* required for the young cohorts and the *Ulva* required for the pellet formulation.

Table 5.7. Monthly dry weight (DW) urchin feed requirements for conceptual urchin-*Ulva* IMTA system for the three different feed scenarios. While Scenario A and C require fresh *Ulva*, it has been converted into dry mass so values are more comparable. The pellets contain 20% *Ulva*.

Feeding scenario	**Pellets (t DW, 20% *Ulva* inclusion)**	**Total *Ulva* (t DW)**
A (Fresh *Ulva* only)	0.00	2.43
B (Pellets only)	2.57	0.51
C (*Ulva* then pellets)	1.33	0.98

5.3.3.7 *Ulva* nitrogen assimilation and growth

The results of the *Ulva* assimilation and growth model indicated that the 300 m^2 raceway is more than sufficient to remove all the TAN emitted from the urchin system. Scenario A, which has effectively double the TAN input of Scenarios B and C (0.012 mg/l and 0.06 mg/l average respectively), also has effectively double the nitrogen uptake (Fig. 5.12). The spatial TAN uptake for Scenario A ranges between 0.187 to 0.390 g.m^{-2}.d^{-1}, while for Scenarios B and C, this metric ranges between 0.088 and 0.193 g.m^{-2}.d^{-1}. To sustain these values, the total nitrogen uptake for the entire raceway of all scenarios is equal to or exceeds the TAN input from the urchin emission for approximately the first 11 days of the production cycle, where, for example, their total N uptake on day 11 for Scenario A is 57.008 g, while the urchins only provide 41.508 g of N from their TAN emissions. This discrepancy in nitrogen uptake of the *Ulva* and nitrogen supply from the urchins is made up by the internal nitrogen stores within the *Ulva*.

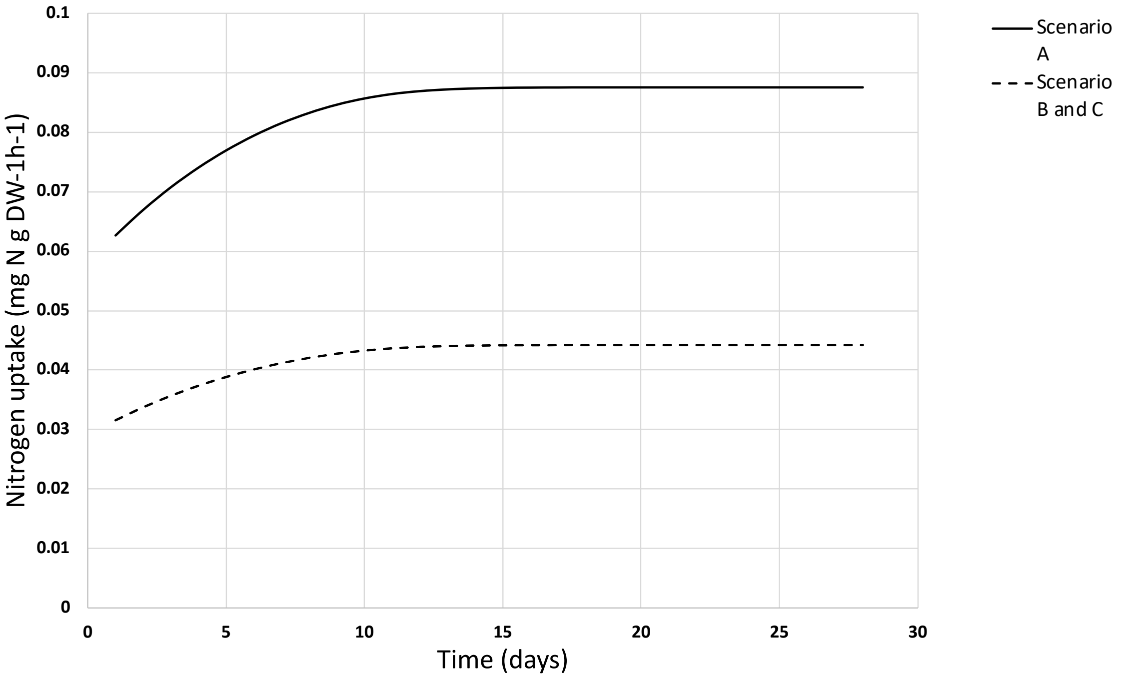


Fig. 5.12. The nitrogen uptake of the *Ulva* in the raceway given the total ammonia nitrogen (TAN) input from the urchin system over the production cycle for the various scenarios. Scenarios B and C are shown as a single curve as they have effectively the same average TAN input.

The *T. gratilla* production system does not provide enough TAN to support *Ulva* growth in a 300 m^2 *Ulva* raceway, regardless of feeding scenario. While the first few days of the production cycle show some increase in biomass, where all scenarios have a maximum net growth of 0.281 (daily SGR), this is not sustained for long. By day 11, there is a net decline in *Ulva* (Fig. 5.13). While Scenario A had nearly double the TAN concentration of both Scenario B and C, neither concentration was substantial and thus had little influence on the *Ulva* performance (Figs. 5.13 and 5.14).

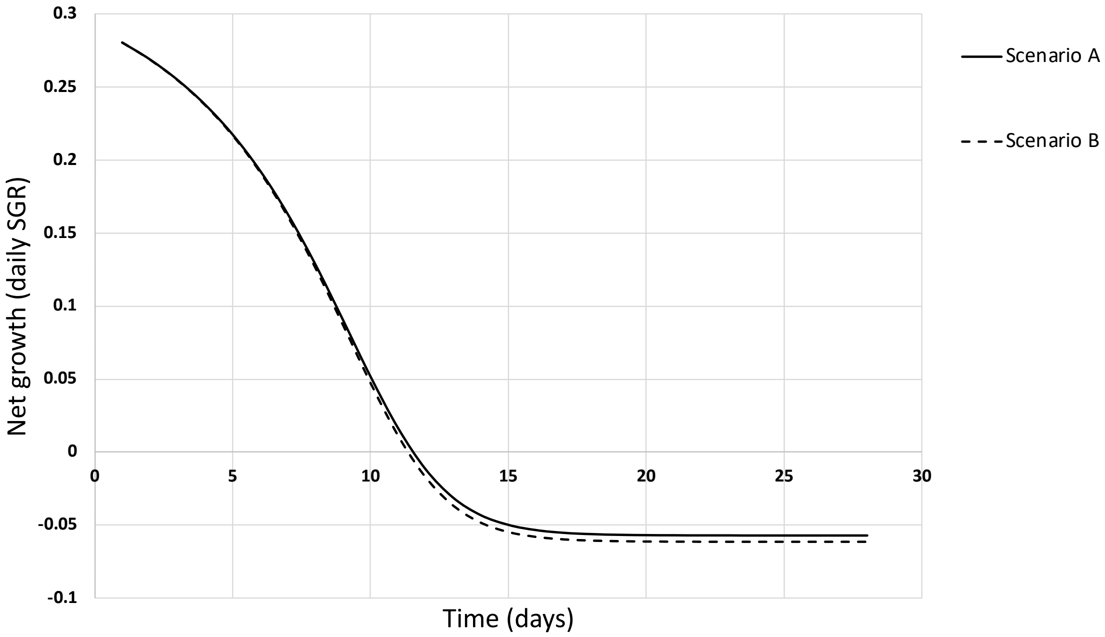


Fig. 5.13. The net daily specific growth rate (SGR) of the *Ulva* for the various scenarios over the production cycle. Net growth is the predicted growth given the nitrogen availability, minus the expected natural daily loss of biomass. Scenarios B and C are shown as a single curve because they have effectively the same average total ammonia nitrogen (TAN) input.

The total *Ulva* biomass increases for the first 11 days for Scenario A and 10 days for Scenarios B and C (Fig. 5.14). Both achieve a total biomass of approximately 57 kg DW, which translates to a stocking density of about 1.4 kg.m^{-2} WW. However, this growth is not sustained and, after 20 days into the production cycle, the *Ulva* biomass is below the initial stocking density. This means that by the end of the production cycle there will be a net loss of *Ulva*. The corresponding curve of the *Ulva*'s internal nitrogen composition (N_{int}; Fig. 5.14) largely explains the rise and decline in *Ulva* growth. This model assumed the initial nitrogen composition of *Ulva* is 20 mg N. g^{-1} DW, and therefore has internal nitrogen stores that can support growth. This changes on day 11 or 10 (for Scenarios A or B and C respectively), where the internal nitrogen content reaches the minimum value of 10 mg N. g^{-1} DW, at which point growth is not supported and the natural biomass loss (Fig. 5.13) results in a net decline in biomass (Fig. 5.14)

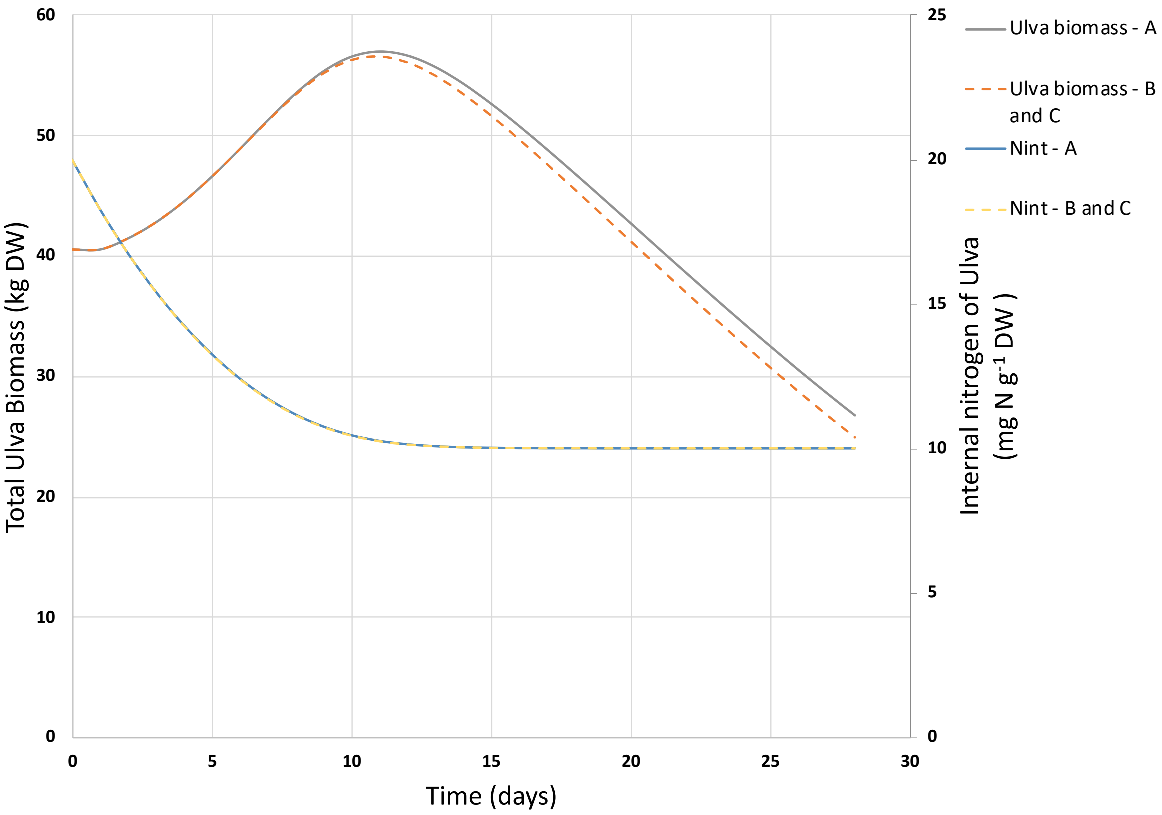


Fig. 5.14. The total biomass of *Ulva* present in the raceway over the culture period is shown on the primary (left) y-axis while the internal nitrogen composition of *Ulva* (N_{int}) is shown on the secondary (right) y-axis. The change in N_{int} over time for all scenarios is the same

To indicate the maximum potential of this *Ulva* raceway, the light limited growth was calculated. If a 300 m^2 *Ulva* raceway is stocked to 1 kg WW.m^{-2} with light levels expected in a tropical region and is not nitrogen limited (only light limited), this would result in the constant specific growth rate (SGR) of 0.299 and yield 362.371 kg DW per production cycle (month) if harvested daily to keep the *Ulva* at the density of 1 kg.m^{-2}. The amount of feed that a 300 m^2 *Ulva* raceway could produce relative to the *Ulva* requirements of the three different feed ratios are 14.91%, 71.05% and 36.98% for scenarios A, B and C, respectively.

5.4 Discussion

5.4.1 The feasibility of the *Ulva* raceway as a biofilter for *T. gratilla* TAN emissions

The *Ulva* raceway could be considered valuable in that it can remove all the TAN provided by the urchins. However, it could also be considered excessive as a biofilter. This raceway would need to remove an average of 0.138 g TAN.m^{-2}.day^{-1} for the feed scenario with the highest TAN emissions (Scenario A). The lowest reported TAN removal of *Ulva* is 0.4 g m^{-2}.day^{-1}, with the same study also reporting the highest value of 7.4 g m^{-2}.day^{-1} (Msuya and Neori, 2008). Thus, while a 300 m^2 *Ulva* raceway would be capable of removing all TAN, it would be highly inefficient in terms of biofiltration, especially in the context of farmland footprint (space utilization) and economics.

Reducing the size of the *Ulva* raceway may not solve this issue. If a conservative TAN removal rate of 2 g m^{-2}.d^{-1} is assumed (based on the literature review of Ben-Ari *et al.*, 2014), a raceway of 20.754 m^2 would be capable of removing the TAN from Scenario A. This is unlikely to be feasible because if other variables, specifically flow rates, remain constant, the turnover rate of the raceway would be 17.202h^{-1}. The turbulence would be severe and, while not tested, it seems unlikely that the *Ulva* would be productive in this environment. Scenarios B and C have effectively half the TAN output of Scenario A. As such, the adequately sized raceway, in terms of TAN removal, would have a considerably higher turnover and be less feasible.

Reducing exchange rates and/or increasing urchin stocking densities may justify the size of the *Ulva* raceway. However, this is not recommended without further experimentation. This study observed that flow rates do not have a linear influence on TAN production, where reducing the flow rate does not have a considerable increase in TAN. Furthermore, the flow reduction and greater stocking densities may reduce urchin production efficiency via negative inter-individual interactions (Chapter 2) and the effects on the system's carbonate chemistry (Mos *et al.*, 2015; Shpigel and Erez, 2020). While *Ulva* could potentially be used as an efficient TAN biofilter for *T. gratilla* in a different system, it would not be effective as the system specified here and provides neither the desired economic nor environmental benefits.

5.4.2 The feasibility of the *Ulva* raceway as a feed source for *T. gratilla*

Due to the lack of nitrogen supplied by the urchins, there will be a net loss of *Ulva* biomass over the production cycle (Fig. 5.14). This means the *Ulva* raceway in this farm-scale model will not provide a source of urchin feed and therefore does not assist justifying its integration into this aquaculture system. Due to the assumptions of this model, a real farm may better support *Ulva* than is predicted by this model. However, it is unlikely that the distinction would be substantial. Most importantly, there is evidence the *Ulva* raceway could provide substantial quantities of *Ulva* feed relative to urchin requirements if an additional nitrogen source was provided or if there was more efficient use of the feed-derived nitrogen waste from the feed.

The light-limited *Ulva* production submodel indicated the production could be 0.362 t DW per month if nitrogen was not limited. This production rate (43.139 g DW $m^{-2}\,d^{-1}$) is very similar to what is observed in real systems (Ben-Ari *et al.*, 2014; Mata *et al.*, 2010; Mata and Santos, 2003; Msuya and Neori, 2008; Neori *et al.*, 1991). This production could be increased with stocking density optimisation; however, this is beyond the scope of this thesis. While the estimated *Ulva* production would not completely fulfil the *Ulva* demand for any of the feeding scenarios (Table 7), it would still supply relatively substantial amounts, up to 71.05% (for Scenario B), thus providing economic and environmental benefits. This implies the most practical feeding scenario is scenario B, where only the formulated feed (with 20% *Ulva* inclusion) is supplied to all urchin cohorts. Furthermore, this feeding scenario will supply considerably more marketable product (Fig. 5.11). Regardless of feed scenarios, *Ulva* may need to be sourced off-site. This would be difficult logistically, especially for fresh *Ulva*. If there are no spatial or nitrogen limitations, *Ulva* production area could also be increased on-site to achieve the required biomass. Alternatively, the pellets could be formulated with an *Ulva* inclusion of 14.09%, instead of the recommended 20%. There is evidence that this would not affect urchin production (Cyrus *et al.*, 2014). While it could be highly beneficial to achieve this *Ulva* production of

0.362t DW per month, the system would require at least 7.24kg of nitrogen per month (where $N_{int} = N_{crit}$).

The model assumes that the only form of nitrogen utilised by *Ulva* is TAN (Section 5.2.6). However, some may argue that nitrate may also be utilised. If it were, it appears unlikely it would have considerable influence on *Ulva* production. Chapter 4 found no evidence that urchins fed pellets emitted any nitrate. However, urchins fed *Ulva* did emit nitrate. As a crude calculation to show the potential of this nitrogen source, it was found that urchins fed *Ulva* emitted nitrate-nitrogen at a rate of 0.00365 mg $N\text{-}N0_3$. g urchin $WW^{-1}.day^{-1}$. That means the entire urchin system could produce a maximum (Scenario A, final week of production cycle) of 48.18 g $N\text{-}N0_3.day^{-1}$. While this is slightly greater than the TAN-N emissions, it is only 18.63% of the nitrogen required to sustain the light-limited production. Regardless of the *Ulva*'s ability to simultaneously assimilate TAN and $N0_3$, there is no evidence the *T. gratilla* effluent will be able to support substantial *Ulva* production.

This model has not considered any additional sources of dissolved inorganic nitrogen (DIN, which includes both TAN and nitrate). This effectively makes the assumptions that the recirculation rate is 100%, meaning no fresh seawater is added. This 100% recirculation is seldom practised in commercial aquaculture and this study is not necessarily recommending it. This assumption was made to simplify the model and to increase its applicability (discussed in Section 5.2.6). A *T. gratilla-Ulva* farm might be capable of supporting *Ulva* production if fresh seawater with a high level of ambient DIN is pumped into the system. However, this study cannot recommend relying on oceanic DIN as a primary source of nitrogen because it is likely to fluctuate and unlikely to supply enough nitrogen for substantial growth (Gavio *et al.*, 2010; Glenn *et al.*, 1999; Rees *et al.*, 1999). Even in the high nutrient temperate waters of the South African West Coast, *Ulva* paddle raceways supplied directly with seawater need to be supplemented with an extra nitrogen source to achieve high growth levels (Shuuluka *et al.*, 2012).

Given the feed requirements and the protein contents of the feed (*Ulva* assumed $N_{int} = N_{crit}$), the daily nitrogen inputs of the feed added to the urchin system is 1.739, 3.780 and 2.652 kg $N.d^{-1}$ for Scenarios A, B and C respectively. This indicates that the quantity of nitrogen derived from the feed should be sufficient to support substantial *Ulva* growth. The TAN derived nitrogen predicted to be emitted from the urchin system only accounts for 2.387, 0.548 and 0.862% of the inputted feed nitrogen. As intended, the urchins will retain some of this nitrogen. It is not known how much will be retained by *T. gratilla*, but *Paracentrotus lividus* has been found to have a nitrogen retention from 3.14% to 10.53% (Lourenço *et al.*, 2020). As such, there is likely to be a considerable amount of nitrogen emitted into the water column. Removal of nitrogen within the urchin tank is possible and has been observed at various degrees in other aquaculture systems via the microbial community (Fu *et al.*, 2015) along with/or denitrification (Hargreaves, 1998; van Rijn *et al.*, 2006). However, Chapter 4 did not provide clear evidence of any nitrogen removal by the tank, suggesting if the urchin tank does remove nitrogen, it may not be to a great extent. The most likely nitrogen sink of this system is in the particulates. The accumulation of settled particulates (sludge) was shown to have a strong influence on nitrogen emissions (Chapter 4). The nitrogen apparent digestibility coefficient (ADC), i.e., the amount of nitrogen found in the faeces relative to the amount of nitrogen in the feed, for *T. gratilla* consuming the 20U pellet has been found to be 71.2% (Cyrus *et al.*, 2014). This metric only accounts for settled particulates and not suspended particulates. Therefore, it is likely that the total amount of nitrogen in solids within the environment exceeds this ADC value. This indicates the likely pathway for the unaccounted feed-derived nitrogen.

Ulva cannot assimilate solids. Therefore, nitrogen in this state cannot be utilised and, if dealt with incorrectly, could negatively impact the environment. A mitigation strategy for this issue is unclear. This could perhaps involve positioning the outflow of the urchin tanks at the bottom of the tank instead of the surface to transfer the settled particulates into the *Ulva* raceway. The turbulence experienced by the sludge may release nitrogen, which could be utilised. Further investigation is required because, while there is evidence of sufficient nitrogen to support substantial *Ulva* growth to be a significant feed source for *T. gratilla*, this nitrogen is in the incorrect form.

When compared to observed TAN concentrations in abalone effluent, the predicted (and observed) concentration of TAN in urchin effluent is considerably lower. The mean TAN concentration of effluent from tanks stocked with *H. midae* (at densities and flow rates similar to those on commercial farms) was found to be 0.048 mg/l using feed with the same protein content as the pellets used in this study (Naylor *et al.*, 2014). This is approximately five times greater than the TAN levels expected from *T. gratilla*. The reasons for this are not clear but may be linked to nitrogen emissions of urchins being primarily as particulates and not dissolved. This suggests the conceptual IMTA system described for this study is designed for and well suited to culturing *H. midae* but may not be for *T. gratilla* due to differences in nitrogen discharge between these species.

5.4.3 Potential for TAN accumulation within the *T. gratilla* system to reduce urchin production

There is evidence that TAN levels would not negatively impact urchin production on the conceptual farm. The maximum predicted TAN concentration in an urchin tank across all feed scenarios was 0.018 mg/l. To estimate a worst-case scenario, one could assume this maximum TAN concentration occurred concurrently with a high pH 8.5, thus resulting in a free (un-ionised) ammonia nitrogen (FAN) concentration of 0.002 mg/l. This is well below the FAN (0.016 mg/l) level that reduced growth (but with no mortalities) of S. *droebachiensis* (Siikavuopio *et al.*, 2004b), which is the best indication available of ammonia toxicity of urchins. Therefore, it can be concluded that there is no evidence *T. gratilla* production will be reduced by ammonia in this aquaculture system.

It is important to note that while nitrogen accumulation is unlikely to reduce urchin production, there are other water quality parameters, beyond the scope of this model, that may reduce production. Carbon dioxide specifically is most likely to be limiting (Mos *et al.*, 2015; Shpigel and Erez, 2020). The extent of this limitation could be modelled, and various strategies could be taken to reduce its effects. One of these strategies could be the biofiltration of *Ulva*. Regardless of the carbon chemistry, there is evidence the production of urchins in this conceptual farm is realistic because these specific conditions (stocking densities, flow rates etc) have been tested empirically in this thesis and in the literature.

5.4.4 Estimation of *T. gratilla* production

This *T. gratilla-Ulva* IMTA system is estimated to produce 0.42 and 0.73 t of urchin gonad per month if fed *Ulva* and formulated feed as a finishing diet respectively (Fig. 5.11). The total predicted annual harvest of whole urchins is 38.64 t. AusUni Pty Ltd, the only described commercial *T. gratilla* production facility (no longer in operation), was reported to have capacity to produce 12 t of whole urchin per annum (Brown and Eddy, 2015). The dimensions and number of tanks and baskets are remarkably similar to those of this conceptual farm. On closer inspection, it appears that the reported yield of this farm may be incorrect. It is likely to be 12 t per harvest. The reported stocking densities (which were greater than those suggested in this study) result in each of the 16 “roe conditioning” tanks having a net biomass of approximately 12 t. To be conservative, it is assumed it took a year for them to achieve market size, which is approximately double the time observed in other studies (Cyrus *et al.*, 2014; Dafni, 1992; Shpigel *et al.*, 2018). The report implies there are at least three cohorts at one time and therefore it can be assumed that the true annual yield would be three times the reported “annual” production. It is thus assumed that the true annual yield may be 36 t, which is very near the 38.64 t predicted to be produced by the conceptual farm in this study.

When compared to the aquaculture of other benthic species, production of *T. gratilla* with in this system appears high. This conceptual IMTA farm occupies 1 200 m^2 of land area (including the *Ulva* raceway and spaces between tanks) and the spatial gonad production would be circa 42 and 73 t $WW.ha^{-1}.year^{-1}$ for the finishing diets of *Ulva* (Scenario A) and pellets (Scenario B and C) respectively. This yield of marketable product is high when compared to sea cucumber farming. Sea cucumbers cultured in ponds are reported to yield 3 t $DW.ha^{-1}.year^{-1}$ (Brown and Eddy, 2015) and sea cucumber ranching beneath mussel beds is expected to yield 0.75 t $DW.ha^{-1}.year^{-1}$ (Brown and Eddy, 2015). A more direct comparison can be made with abalone, which are cultured in effectively the same (intensive) system. The predicted production of whole urchins per water surface area (farm area occupied by urchin tanks) is 323.26 t $WW.ha^{-1}$ while whole abalone production is calculated to be 135.29 t $WW.ha^{-1}.year^{-1}$ based on values from Cloete (2009). This suggests *T. gratilla* farm in intensive systems could achieve much greater yields relative to other high valued invertebrates, this is likely due to the high growth rates of *T. gratilla*. However, market research and economic feasibility analysis is required before this species can be deemed financially lucrative.

5.4.5 Improvements

As might be argued in the case of all models, this model could be more sophisticated. Also applicable to the model is the statement, "the key to a model's usefulness is leaving out the unimportant factors and capturing the interactions between the important factors" (Ford and Ford, 1999). Importantly, the model has achieved its goal and objectives with a majority of the submodels being validated within this study (in the case of the urchin module) or in other studies (for the *Ulva* module). It appears very unlikely a more sophisticated and/or accurate model would change the conclusions drawn here.

However, if this model was used for different objectives, particularly those related to production optimisation, the following improvements are recommended:

- As mentioned, the carbonate chemistry is most likely the first parameter to limit *T. gratilla* production. If a model was developed to predict the increase of $C0_2$ and decrease of bicarbonates in a *T. gratilla* aquaculture system it could greatly assist with determining optimal flow rates (volumetric), stocking density and necessary mitigation strategies (such as *Ulva* biofiltration).
- Microbial communities have been shown to have a strong influence of nutrient loads in aquaculture (Bentzon-Tilia *et al.*, 2016) and there is evidence IMTA can modulates the microbiome in a manner which will enhance production (de Jager, 2021; Macey *et al.*, 2022). The impact of the microbiome is an important potential benefits in IMTA and could be modelled to quantify (Fu *et al.*, 2015).
- There is evidence that the regression model provides accurate predictions of TAN emissions, even from tanks of a considerably larger scale (Section 5.3.2.2). However, it is still advised to continuously validate this model with external scale-appropriate data, especially if these data can be sourced from a functioning *T. gratilla* farm. Furthermore, additional training data can be added to this GAM model to further increase its accuracy and applicability.

- There is a significant amount of natural and measurement variation within the urchin growth metrics (Chapters 2 and 3). As such, inter-individual variability functions in both urchin production submodels should be introduced. This could be beneficial, especially for the growth model since it would allow for better estimations of the number of urchins that should be culled due to being slow growers. This would allow for more accurate seed requirement predictions. Furthermore, more calibration of the growth model would be beneficial. Net underprediction of this growth model suggests true stocking densities may be greater than predicted. While this may result in greater than expected production, this could also have detrimental effects if it was used for system optimisation because TAN levels could be greater than expected and more biofiltration could be required. This thesis has not focused on optimisation but rather feasibility, where even if the stocking density was 13.11% higher, it would still not support considerable *Ulva* growth. Thus, for these objectives it does not appear necessary to reinvent the wheel with new production models as none of the major conclusions would be different.

5.4.6 Conclusion

This farm-scale model provides evidence that this conceptual farm is not feasible. However, it also indicates that an *Ulva*-*T. gratilla* farm, if designed appropriately (not as for abalone), could be viable. The primary limitation identified in this study is a lack of useable nitrogen to support *Ulva* growth. This subsequently prevents justification of the *Ulva* raceway. However, there is sufficient nitrogen for the *Ulva* growth inputted into the system through the urchin feed (in all feed scenarios)—despite this being in the incorrect form (solids). This is not only an inefficient use of nitrogen but could also have negative environmental impacts if discharged. Therefore, to make this IMTA system achieve its intended primary environmental and economic objectives, it is necessary to determine a means to transform this nitrogen into a form which can be utilized by *Ulva*. Once this occurs, there appears to be much promise from this IMTA system, particularly given that the production rate of *T. gratilla* is very high compared to similarly valuable invertebrates in aquaculture. These findings were revealed by the farm-scale model by consolidating extensive knowledge, which brought us closer to understanding what is required to integrate *Ulva* and *T. gratilla* successfully and sustainably in commercial aquaculture. The model could be used as the foundation for the development of further site-specific economic and environmental feasibility analyses and lifecycle assessments. This tool could play a pivotal role in the establishment and growth of this sector.

Chapter 6: General conclusion

The aim of the thesis was to use a farm-scale model to assess the feasibility of a recirculating, land-based system that integrates *Tripneustes gratilla* and *Ulva*. Prior to this, specific literature and data gaps required for the model were addressed. Specifically, the study determined the influence of basket depth on *T. gratilla* production (Chapter 2), suggested an appropriate stocking density (Chapter 2), developed a protocol for measuring live urchins accurately on a large scale (Chapter 3), and investigated the impact of *T. gratilla* on water chemistry in an aquacultural context (Chapter 4). These findings, along with much literature, were conglomerated in a farm-scale model (Chapter 5) to assist the establishment of a new and sustainable aquaculture industry.

6.1 Basket depth optimization

The major finding of the initial experiment provided evidence that the lower production of urchins cultured in deeper baskets is due to reduced consumption. This finding has ramifications extending beyond *T. gratilla* production as it solves this question asked by many researchers of various urchin species (Christiansen and Siikavuopio, 2007; Daggett *et al.*, 2006; Devin, 2002; Pearce *et al.*, 2002; Siikavuopio, 2009; Siikavuopio *et al.*, 2007). While this finding implies that shallow baskets (approximately 15 cm deep) are likely optimal for maximizing individual growth, it must be acknowledged that shallow baskets may not be optimal for farm production (in terms of biomass yield or economics) or management/maintenance practices. Several pilot farms have experimented with various design of shallow urchin culture systems (Daggett *et al.*, 2006; Devin, 2002; Shpigel *et al.*, 2018). While these systems will likely increase urchin growth, it is probable their capital expenditures (CAPEX), design complexity and management will be considerably more intensive than the simple tank system containing deep baskets (as described in Chapter 5). A specific comparison between these different systems should be conducted where not only individual growth is considered, but also net production and economic metrics. As this was beyond the scope of this thesis, it was decided to model deeper baskets due to the before mentioned reasons and to enhance the similarity of this proposed system to the existing commercial abalone-*Ulva* systems, thus hopefully improving industry adoption via familiarity.

6.2 Stocking density optimization

The stocking density is conservatively and broadly suggested to be kept at approximately 20% coverage given the objectives (grow-out and gonad enhancement) and assumed lack of resource limitations. Realistically, a *T. gratilla* production facility will have certain resource limitations. Therefore, calculations to determine their site-specific optimal stocking density are required. For example, when the supply of urchin juveniles and/or feed is restricted or expensive, lower stocking densities will increase profit as it will maximize the potential of each urchin. On the other hand, if there are no limitations on juveniles and/or feed is inexpensive, but there are constraints on the running costs and/or available space (such as the number of baskets), higher stocking densities may be utilized. Therefore, each facility should determine their own optimal stocking density. However, the 20% coverage, as advised by this study, is an appropriate and conservative starting point for *T. gratilla* culture. While there is anecdotal evidence that urchins smaller than those used in this stocking density trial (< 50 mm test diameter) should perform well at 20% coverage (personal observation), this should be examined and optimized empirically. It is also important to note that while greater densities may produce higher yields on paper, it will increase risk of catastrophic events resulting in mass mortality, such as disease breakouts or system malfunctions. This risk, and the difficulty of accounting for it, is one of the primary reasons for failure for many aquaculture facilities and investor hesitation (Beach and Viator, 2008). Based on these stocking density trials and the general management of *T. gratilla* over the past few years, 20% coverage does not appear to be of high risk.

6.3 Live sea urchin measurement techniques

These production optimization trials required the measurement of thousands of live urchins. This revealed how the traditional techniques used for measuring urchins were highly inaccurate, time-consuming, and completely impractical in a commercial context. Therefore, this would be a bottleneck in any urchin production industry and needed to be removed. The standardized, efficient and accurate protocol on measuring live urchins, developed in Chapter 3, can be used in a research or commercial context for most sea urchin species. There is room for future development, particularly in industry. This software and protocol could be incorporated into various applications, such as a grading machine, making the entire process of urchin quantification and sorting fully automated, reducing labour costs, and increasing efficiency. This could make sea urchin aquaculture more profitable and sustainable in the long term. Furthermore, this is yet another example of how computer vision can be a useful tool in aquaculture (Vo *et al.*, 2021; Yang *et al.*, 2021; Zion, 2012).

6.4 The influence of *T. gratilla* on water chemistry

The initial objective of the experiment conducted in Chapter 4 was to generate appropriate training data to create a *T. gratilla* nitrogen emission submodel for the farm-scale *T. gratilla-Ulva* IMTA model. This objective was achieved, and in the process substantial and novel insight was gained regarding the influence *T. gratilla* has on water chemistry, specifically when fed *Ulva* or the specific formulated feed. This newfound knowledge has important implications for management practices, such as the regular removal of settled solids (sludge). In addition, the study found no compelling evidence to suggest that effluent from *T. gratilla* would have any significant environmental impacts, even if released without remediation. Nonetheless, the study does not advocate for this practice, and this statement does not extend to settled solids. Further research is necessary to fully understand the potential environmental impact of *T. gratilla* solid emissions. This research is a valuable addition to the growing body of evidence that urchin production is limited by carbon chemistry rather than nitrogen toxicity, unlike other aquaculture sectors (Hargreaves, 1998). Thus, the development of dissolved carbon dioxide and pH mitigation strategies is crucial to boost urchin production. A possible carbon remediation strategy could be integration with *Ulva*, as indicated in abalone-*Ulva* systems (Lester, 2021).

6.5 Modelling total ammonia nitrogen (TAN) emissions from *T. gratilla* aquaculture

A black box approach was applied to accurately predict TAN emissions from *T. gratilla* aquaculture systems via an empirical regression model. This is a relatively novel and innovative method compared to the traditional mechanistic models, which may have difficulties creating accurate predictions due to the complex and numerous factors involved in the nitrogen cycle (Pretorius, 2020; Wheaton *et al.*, 1994; Yu *et al.*, 2021). This empirical regression model predicts TAN emissions based on the appropriate data (from Chapter 4) and only required relatively few independent variables, which represent the factors influencing TAN emissions that can be controlled by the system manager, such as stocking density, flow rate, feed quantity and sludge removal. Due to the non-parametric nature of these data, a generalized additive model (GAM) was found to be a highly suitable, flexible and powerful tool for this form of modelling. The scale-appropriate, external validation data demonstrated the high accuracy of the *T. gratilla* TAN emissions model (Section 5.3.2.2). Furthermore, the accuracy of this (regression) model will continue to improve as more data is added. This approach could be applied in other forms of aquaculture, such as pond aquaculture, where similar controllable factors are present.

6.6 Required nitrogen mineralization for the biotechnical feasibility

The farm-scale *T. gratilla*-*Ulva* IMTA model provides evidence that constructing such a system based on the existing commercial recirculating abalone-*Ulva* IMTA systems (as described in Section 1.1) may not be as beneficial as previously thought, however, these benefits may be achieved via system adjustments. The model suggests that while the *Ulva* raceway could remove 100% of TAN emitted by *T. gratilla*, it's not efficient as a biofilter due to its excessive farm footprint (Section 5.4.1). The projected TAN emissions from the *T. gratilla* production system cannot sustain an *Ulva* population, let alone enable substantial *Ulva* production for *T. gratilla* feeding (Section 5.4.2). However, the quantity of nitrogen within the settled solids from the *T. gratilla* production system would likely be sufficient to sustain substantial *Ulva* growth, if it could be converted from solid to dissolved and bioavailable form (mineralised, Section 5.4.2).

Achieving sufficient nitrogen mineralisation does not seem impossible but the exact methodology required for marine aquaculture lacks literature and is unclear. The solution to this issue could be as simple as directing the urchin's settled solids into the *Ulva* raceway, instead of pumping it out of the system. The heterotrophic bacteria present in the raceway could decompose the nutrient-rich volatile organic matter via oxidation, which constitutes 50-90% of aquaculture sludge (Mirzoyan *et al.*, 2010). This would result in mineralisation of organic nitrogen into bioavailable dissolved nitrogen, which could be utilised by the *Ulva*. Additionally, this approach could help reduce the environmental impact of the sludge. While the rate of mineralisation that would occur in the *Ulva* raceway is uncertain, it could be relatively high due to the oxygen enhancement provided by the *Ulva*. Mineralisation in marine aquaculture systems has not been extensively studied, but a study conducted on earthen marine fishponds revealed an average rate of 0.252 g N m^{-2}. d^{-1} (Blackburn *et al.*, 1988). This rate is substantially higher than the mineralisation rate observed in natural systems (Ferguson and Eyre, 2010), but it may not be sufficient to support *Ulva* growth within the farm-scale model. At this rate, the 300 m^2 *Ulva* raceway would only be able to mineralise 75.6 g N. d^{-1}, which is not substantial (less than 5%) relative to the amount of nitrogen added by the urchin feed (at least 1.74 kg N.d^{-1}, as discussed in Section 5.4.2). Although the limited literature does not provide sufficient evidence to support the occurrence of mineralisation within the *Ulva* raceway, further investigation is warranted.

Alternative methods for converting nitrogen in the urchin sludge into a bioavailable form may exist. Up flow anaerobic sludge blanket and expanded granular sludge bed reactors have been successful in accomplishing this in aquaponics (freshwater), with high-nutrient effluent being diverted back into the system to support plant growth (Delaide *et al.*, 2018; Goddek *et al.*, 2018; Monsees *et al.*, 2017). This process offers numerous benefits, including the valorisation of nitrogen and other nutrients, reduction of total suspended solids, reduction of chemical oxygen demand, biogas production, and remediation of the sludge, which can be a major issue in aquaponics (Goddek *et al.*, 2018). Sludge from marine land-based recirculating aquaculture systems, such as the *T. gratilla-Ulva* system described, poses a significant threat to the environment as this aquaculture method grows globally (Mirzoyan *et al.*, 2010). Due to the sludge's salinity content, a practical method of disposal or valorisation has not yet been well established. Consequently, significant research has been conducted into the methodology for dealing with marine sludge, specifically by using it as feedstock for biogas production. However, the research has not been highly successful yet, with generally poor biogas yields due to high salinity and non-adapted inocula (Goddek *et al.*, 2018). Although there is no specific research into using biogas reactors to mineralize nitrogen in marine sludge, indications suggest it could work for phosphate (Zhang *et al.*, 2013). Further research is required to determine whether this could be an appropriate method of mineralising nitrogen from urchin sludge. However, it is suggested that the simpler, previously mentioned method of in-situ mineralisation should be investigated initially.

6.7 Should *T. gratilla-Ulva* IMTA be pursued and what next?

Despite the challenges that need to be addressed, pursuing the *T. gratilla-Ulva* integrated multi-trophic aquaculture (IMTA) system remains a promising endeavour, as indicated in this thesis. If the nitrogen in urchin feed could be mineralised into a bioavailable form for the *Ulva*, this system could become highly efficient in both environmental and economic contexts. Additionally, the *Ulva* could address a major limiting factor of *T. gratilla* production, carbonate chemistry (Lester, 2021). Most fundamentally, the farm-scale model demonstrates the highly impressive production rates of *T. gratilla* compared to other high-value aquaculture species. The gonad production could be circa 73 t WW.ha^{-1}.year^{-1}, suggesting the potential for a lucrative industry.

However, before establishing the industry, attention must be given to two critical stages of *T. gratilla* production: consistent supply of larvae and market development. Despite the successful closure of the *T. gratilla* lifecycle in various research facilities globally, achieving it consistently and at the required scale has not been achieved. Even the largest and oldest known *T. gratilla* hatchery recently announced that it had produced a total of one million juveniles (Department of Land and Natural Resources, 2023). This equates to approximately 7 000 individuals per month, which falls well short of 67 500 weaned urchins (10 mm test diameter) per month required to achieve the production cycle created for the farm-scale model. Furthermore, while there is some evidence of high prices for *T. gratilla* in the Japanese market (Sonu, 2003; “Tsukiji-Market,” 2016), this has not been confirmed empirically. Additionally, a study has shown the difficulties of introducing a foreign urchin product into Japan (Stefansson *et al.*, 2017). This confirmation of market could be achieved via gonad enhancement of wild caught *T. gratilla*. Therefore, prior to establishing a full-scale *T. gratilla* production facility, it is suggested that focus is brought onto achieving scale and consistency of *T. gratilla* larval supply and establishment of market channels.

6.8 Conclusion

This thesis has shown that a recirculating, land-based system that integrates *T. gratilla* and *Ulva* through a farm-scale model is a potential avenue for sustainable aquaculture. The study addressed specific literature and data gaps required for the model. It also determined the influence of basket depth, suggested an appropriate stocking density, developed a protocol for measuring live urchins accurately on a large scale, and investigated the impact of *T. gratilla* on water chemistry in an aquacultural context. The farm-scale model suggests that the *T. gratilla* production capacity of the 42 tanks could result in a monthly gonad harvest between 0.31 and 0.88 t. While there are some limitations in the integration of *Ulva* with *T. gratilla* production, the nitrogen retention model provides evidence that there is potential to create a circular and efficient IMTA system if designed and managed correctly. This study could greatly assist establishing this potential industry, and the model could be used as a tool for environmental impact assessors, farmers, entrepreneurs and investors to design farms or conduct economic, environmental and social analyses across various scales.

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Appendices

Appendix A

While the residuals clump slightly on the higher and lower sides of the predictor variable (Fig. A.1), homoscedasticity can be assumed due to a non-significant Levene's test ($F_{(5,12)} = 0.447$, $p = 0.807$). A higher likelihood of spine loss in tank 1 is apparent (Fig. A.2). Yet, when tank was added as a blocking factor into the model, it was not a significant factor. The data was logged to a base of 10 to attain normality (Figs. A.3).

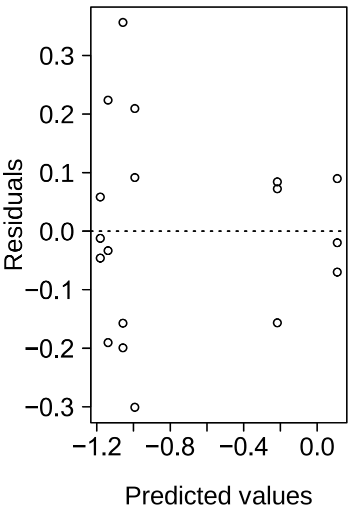


Fig. A.1. A residual plot of the difference between the observed value of the dependent variable and the predicted value.

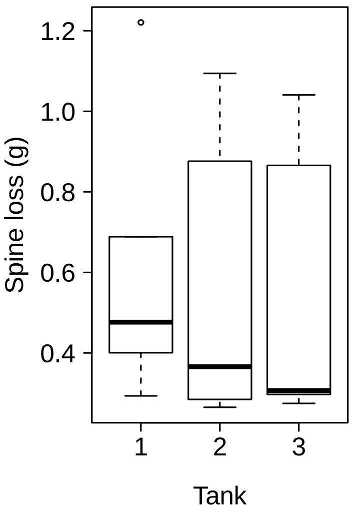


Fig. A.2. Box plot demonstrating the lack of influence the different tanks had on spine loss for *T. gratilla*.

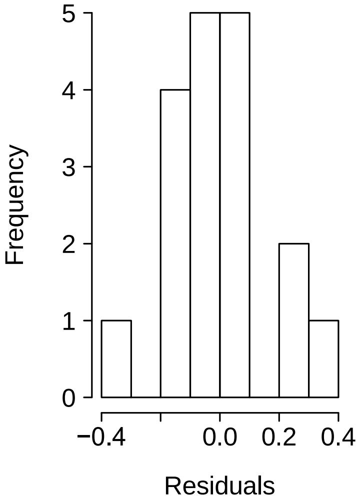


Fig. A.3. A histogram of residuals showing normal distribution.

APPENDIX B

Homoscedasticity was met to a degree (Fig. B.1) and there was no evidence to suggest that the variance in spine loss was statistically significantly different for each of the treatment groups (Levene's Test: $F_{(5,12)} = 2.043$, $p = 0.144$). A box plot demonstrates that the different tanks did not have an apparent effect on the results (Fig. B.2). When tanks were factored into the model as a blocking factor, it did not have a significant effect on the response variable. As such, it was removed and its lack of significance further demonstrates the blocking factors did not affect spine loss. No outliers were present in the data. The assumption of independence and non-selectivity was met as discussed in the experimental design. A histogram (Fig. B.3) and Shapiro-Wilks tests were used to assess assumption of normality. Spine loss was found not to be normally distributed (Shapiro-Wilks = 0.785, $p < 0.001$). Therefore, the data was logged, which did improve it slightly (Shapiro-Wilks = 0.900, $p = 0.058$).

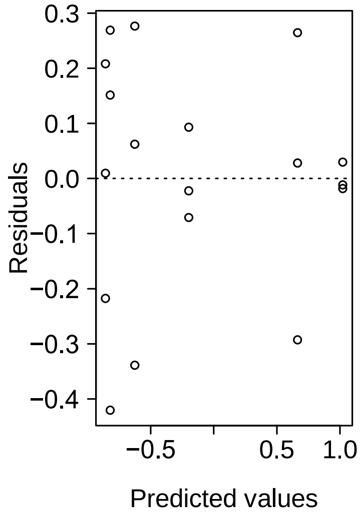


Fig. B.1) A residual plot of the difference between the observed value of the dependent variable and the predicted value.

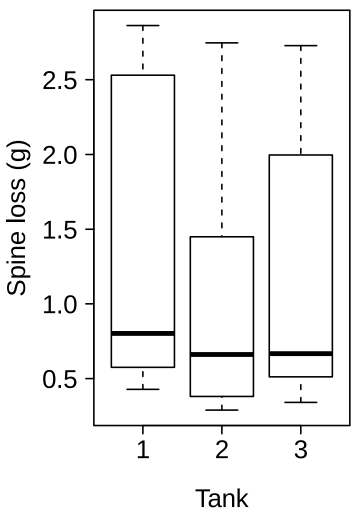


Fig. B.2) Boxplot demonstrating the lack of influence the different tanks had on spine loss for *T. gratilla*.

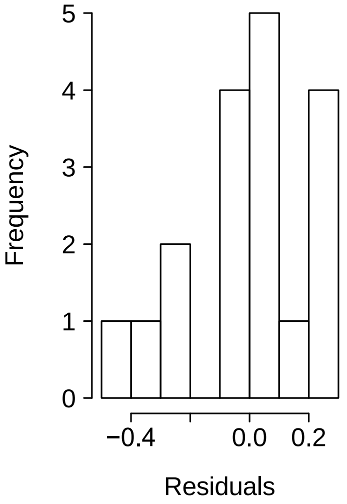


Fig. B.3) A histogram of residuals showing normal distribution.

APPENDIX C

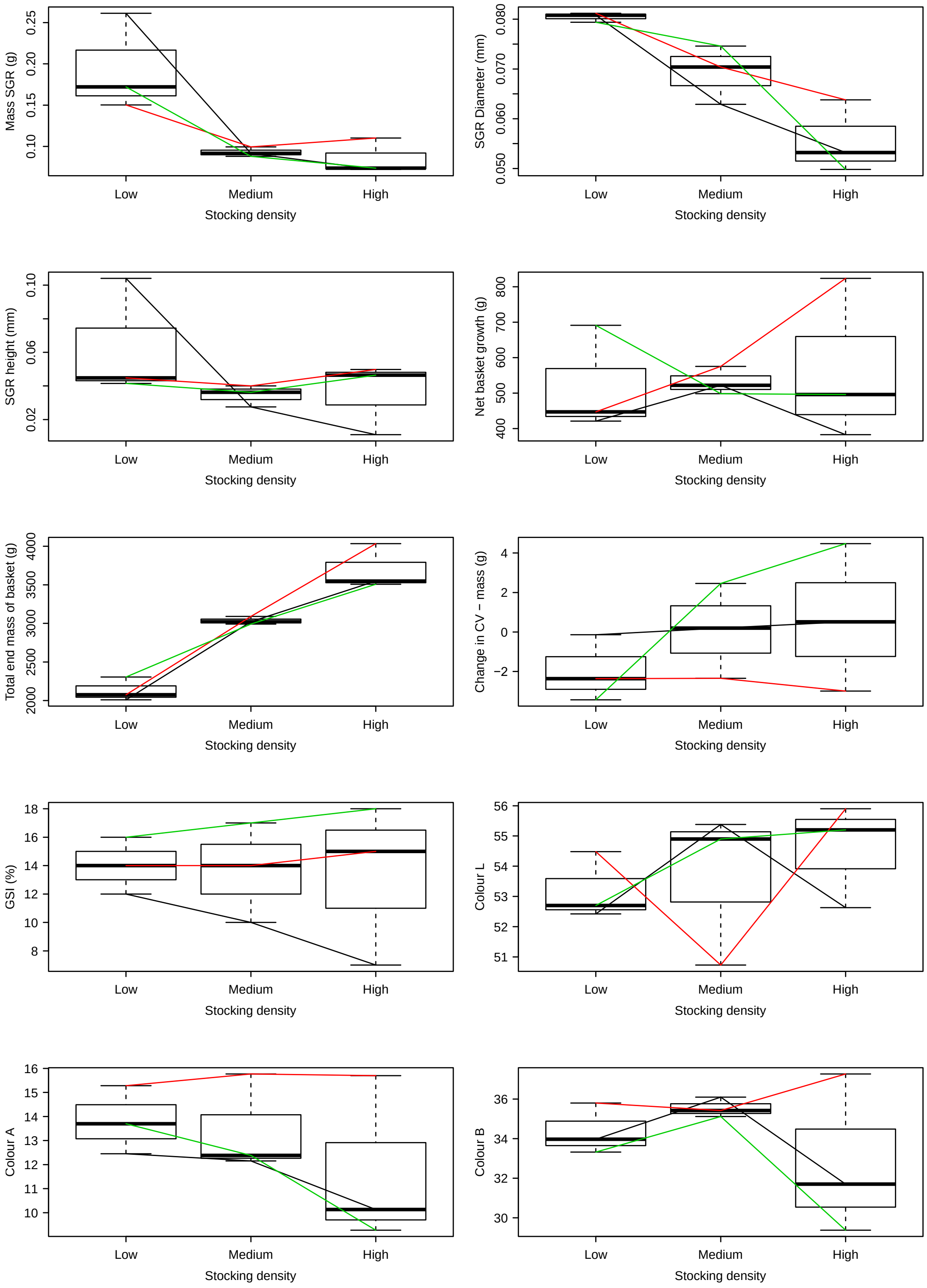
This data set can be assumed homoscedastic as the variance for pellets and *Ulva*-fed deep baskets (2.057 and 84.097 respectively) did not differ greatly to those of the shallow baskets (1.176 and 61.146 respectively). This is confirmed by Levene's test for pellets ($F_{(1,4)} = 0.148$, $p = 0.720$) and *Ulva* ($F_{(1,4)} = 0.054$, $p = 0.827$) experiments. Shapiro-Wilks tests provided no evidence that spine loss from pellet feeding did not meet the assumption of normality (deep baskets, $W = 0.9973$, $p = 0.9007$ and shallow baskets W = 0.99312, $p = 0.8414$). Normality can also be assumed for the *Ulva*-fed experiment (deep baskets - $W = 0.969$, $p = 0.664$ and shallow baskets - $W = 0.858$, $p = 0.262$).

APPENDIX D

Exp.	Parameter	Normality		Similar pop. variance		Additivity		Transformation
		Met	Shapiro-wilks results	Met	Levene's test result	Met	Tukey's 1df result	
1) Grow-out phase experiment	Mass SGR (g)	N	W =0.82, *p* =0.034	Y	F =1.0195 *p* =0.4158	Y	F = 5.242 p = 0.106	Logged (Shapiro-Wilk, W = 0.911, *p* = 0.323)
	Diameter SGR (mm)	Y	W =0.907, *p* =0.299	Y	F =0.956 p =0.436	Y	F = 1.076 p = 0.375	Not required
	Height SGR (mm)	N	W =0.817, *p* =0.032	Y	F =0.415 p =0.678	Y	F = 4.78 p = 0.116	Logged (Shapiro-Wilk, W = 0.884, p= 0.1746)
	Total basket growth (g)	Y	W =0.901, *p* =0.259	Y	F =0.713 p =0.527	Y	F =1.415 p =0.320	Not required
	End basket mass (g)	Y	W =0.934, *p* =0.521	Y	F =0.524 p =0.617	Y	F =1.845 p =0.267	Not required
	Change in CV (%)	Y	W =0.922, *p* =0.413	Y	F =0.5655 p =0.595	Y	F -=4.520 p =0.123	Not required
	GSI (%)	Y	W =0.946, *p* =0.656	Y	F =0.560 p =0.598	N	F =36.632 p =0.009	Not required
	Colour L	Y	W =0.912, *p* =0.330	Y	F =0.212 p =0.814	Y	F =0.186 p =0.695	Not required
	Colour A	Y	W =0.920, *p* = 0.394	Y	F =0.269 p =0.772	N	F =9.371 p =0.055	Not required
	Colour B	Y	W =0.930, *p* =0.4881	Y	F =1.505 p =0.296	N	F =44.193 p =0.007	Not required
2. Gonad enhancement experiment	Mass SGR (g)	N	W = 0.915 p = 0.070	Y	F = 0.105 p = 0.955		NA	Logged (Shapiro-Wilk, W= 0.962, p= 0.563)
	Diameter SGR (mm)	Y	W =0.974 p =0.830	Y	F =1.555 p = 0.236		NA	Not required
	Change in CV (%)	Y	W =0.938 p = 0.198	Y	F =1.159 p = 0.354		NA	Not required
	Total basket growth (g)	Y	W =0.939 p =0.208	Y	F =0.837 p =0.492		NA	Not required
	End basket mass (g)	N	W =0.861 p =0.006	Y	F =0.119 p =0.948		NA	Not truly continuous, therefore not fair to test
	GSI (%)	N	W = 0.911 p =0.059	Y	F =0.643 p = 0.598		NA	Logged (Shapiro-Wilk, W =0.932, *p* =0.150)
	Total predicted gonad yield (g/m^2)	Y	W =0.852 p =0.005	Y	F = 0.725 p =0.551		NA	Logged (Shapiro-Wilk, W = 0.954, *p* = 0.410)
	Colour L	N	W =0.875 p =0.011	Y	F =1.22 p =0.332		NA	Test not applied
	Colour A	Y	W =0.921 p =0.092	Y	F =0.796 p =0.512		NA	Not required
	Colour B	Y	W =0.942 p =0.248	Y	F =1.631 p =0.219		NA	Not required
3.Behavorial interactions	Consumption	Y	W = 0.970 p = 0.795	Y	F = 0.177 p = 0.839		NA	Not required
	Faeces	Y	W = 0.953 p = 0.478	Y	F =1.410 p = 0.274		NA	Not required
	Spine loss	N	W = 0.884 p = 0.031	Y	F = 3.588 p =0.053		NA	Logged (Shapiro-Wilk, W = 0.931, p= 0.204)

An outlier was observed in the data set relating to mass and gonad quantity derived from the same data point (basket) of the gonad enhancement stocking density experiment (Figs. 12, 15, 16 and 17). This point was not removed as it did not appear to be a data collection error and results of Levene's test results did not indicate there were any issues (Appendix E), although this may lower the chance of rejecting the null hypothesis.

Appendix E



Figs. E.1 Box plots of all independent variables of the grow-out phase stocking density trial. The coloured lines of the same colours indicate the same blocks (tanks), this allows for checking the assumption of additivity as described in section 2.2.12.5

Appendix F

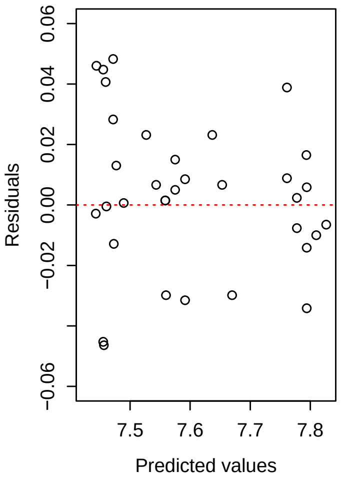


Fig. F.1) A residual plot of the difference between the observed value of the dependent variable and the predicted value.

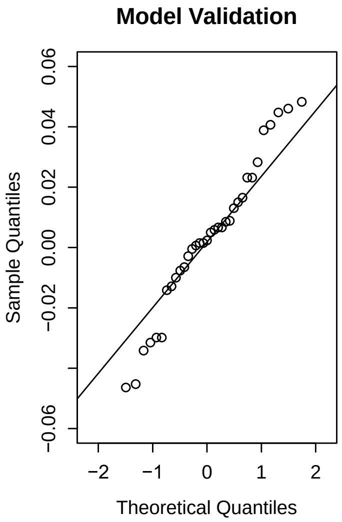


Fig. F.2) QQ plot demonstrating data fits a theoretical distribution by relative to the sample distribution.

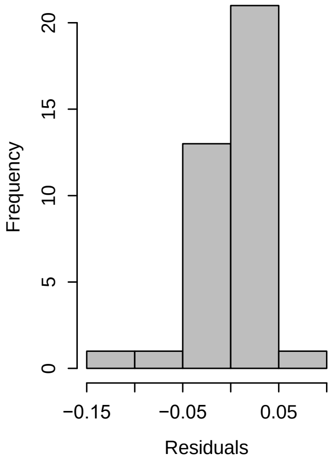


Fig. F.3) A histogram of residuals showing normal distribution.

Appendix G

Protocol: Determining mass and diameter of live urchins

Set up

1. All urchins from each experimental unit can be weighed together, thus an appropriate weight scale (ideally with at least 2 decimal places but depending on the quantity of urchins per treatment) and container must be acquired. The container must be stable on the scale and easy to tare. This could be the photo box described below.
2. Any container could be used for the photo box provided it serves the following functions:
    a. Appropriate size to hold the required number of urchins, with adequate spacing between them (*ca.* 1.5cm).
    b. Somewhat standardises lighting conditions.
    c. Standardises the distance from the camera to the urchins.

   A plain white background is strongly recommended. If a photo box is constructed as used in this study and described below, it will increase the likelihood that the default settings of the computer vision program will be appropriate and in general fewer alternations will be required.
3. To replicate our photo box, attain three ‘fish’ Styrofoam boxes (70cm x 35cm x18cm) and stack them on top of each other, retaining only the lid of the top box. Removed the bottom “floors” of the top two boxes, making a continuous box from the top lid of the upper box to the floor of the bottom box. To allow for the image to be taken, a small hole must be cut in the centre of the lid of the top box. A reference object, with a known diameter and ideally completely black, must be placed on the most left-hand side of the image, and no other objects placed further left of this object.

Acquiring images and mass

1. Slowly remove the urchin basket from the water while gently shaking the basket to ensure urchins detach from the sidewall and fall into the water and remain on the bottom of the basket. Do not allow urchins to fall to the bottom of the basket once it is completely out of the water as this can damage the urchins
2. Remove the basket from the water body and allow it to drip dry for at least 90 seconds before weighing.
3. Remove urchins from the basket and place them into a container that has been tared and record mass.

4. Place urchins into the photo box (or leave them in if this was the container also used to determine mass).
5. It is necessary to have approximately 1.5cm gaps between urchins.
6. Ensure the reference object is on the most left-hand side of the image.
7. Once the reference objects and urchins are placed in the bottom of the container, the lid should be closed, and a mobile phone should be placed on the lid with the camera directly above the central hole
8. The camera should be set to a magnification of 1x without any other filters.
9. The filename of the image must be changed to the name of the group of urchins, this name will be used to label the urchin measurement in the output CSV file.
10. Return urchins to the body of water as soon as possible

Processing images

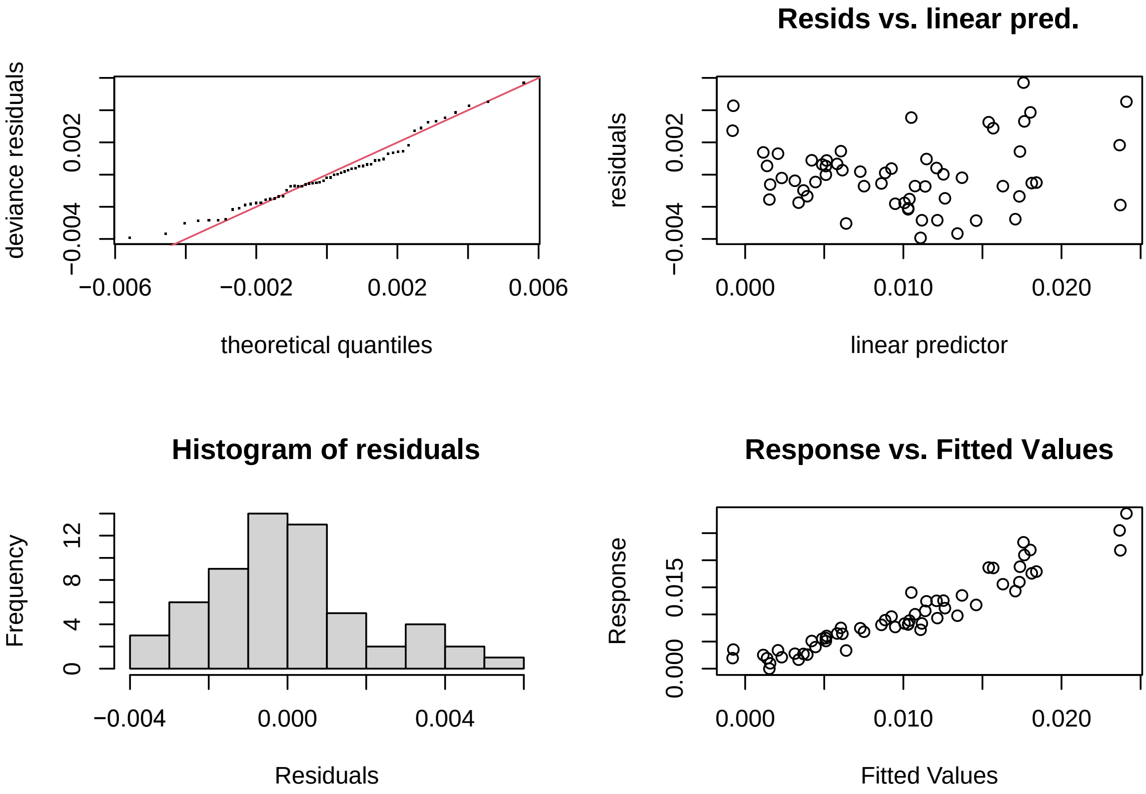
1. The program can be found at https://github.com/TheoBatik/urchinvision
2. This study found that images with a size of approximately 1.2Mb were precise while not requiring too much processing time.
3. Input diameter of reference object for "widths" in the "args" command (line 34)
4. Input image location
5. Run the program, follow prompts and check if contours follow the test of the urchin

Appendix H

Table H.1. The coefficient of variation (CV) and standard deviation (SD) of various measurement techniques for 2 different urchin species and for a single operator (S.O.) and multiple operators (M.O.). The standard deviation could be applied in power analyses for other studies but cannot be used as a comparison of variance between measurement methods.

SPECIES	METHOD	CV (%)	SD
T. GRATILLA	Computer vision	1.55	1.03mm
	Mass S.O.	0.84	0.62g
	Mass M.O.	1.05	0.85g
	Diameter S.O.	2.02	1.11mm
	Diameter M.O.	2.41	1.59mm
	Height S.O.	3.76	1.36mm
	Height M.O.	6.24	2.42mm
	Total basket mass	1.67	6.60mm
P. ANGULOSUS	Diameter S.O.	2.66	0.87mm
	Computer vision	2.06	0.83mm

Appendix I



Figs. I.1. Various internal model/assumption validation plots for the GAM model predicting TAN